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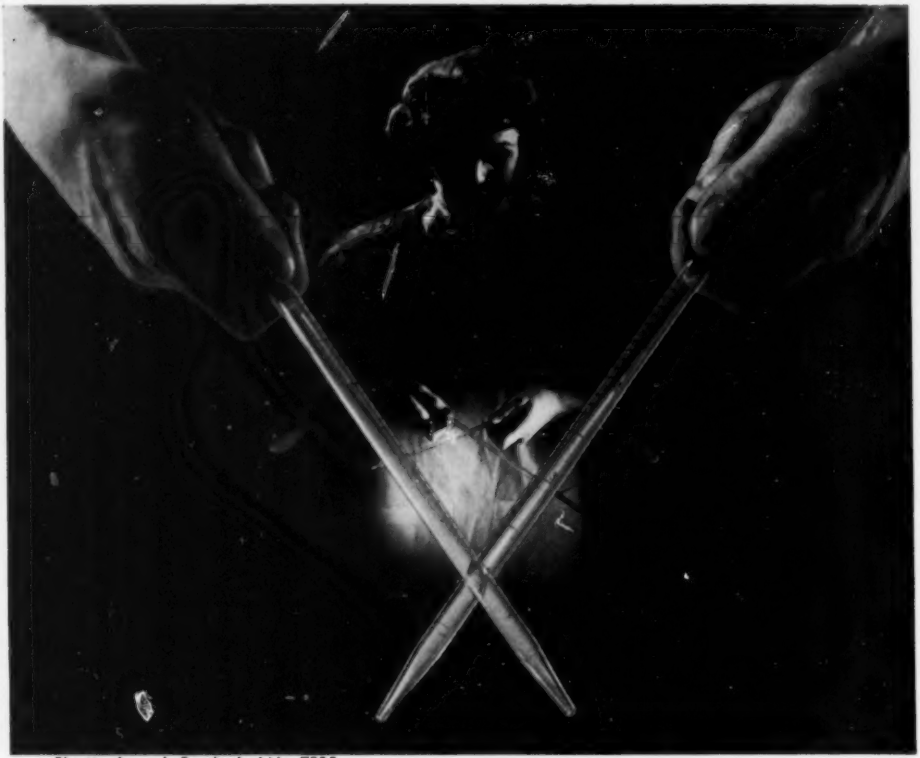

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*Dodd, M. C., and Stillman, W. B.: The in vitro bacteriostatic action of some simple furan derivatives, J. Pharm. Exp. Ther. **82**:11, 1944.

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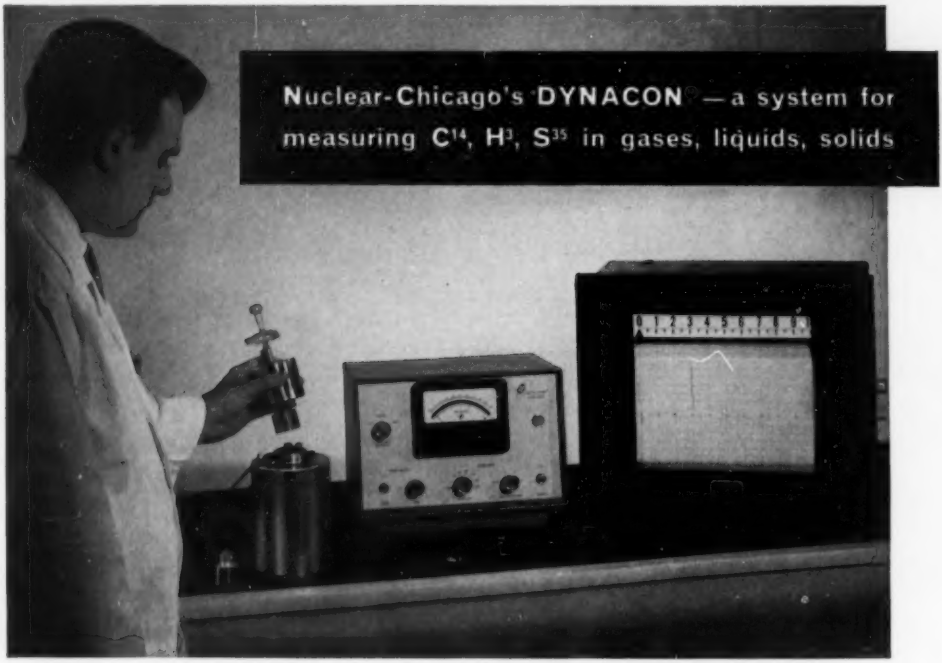
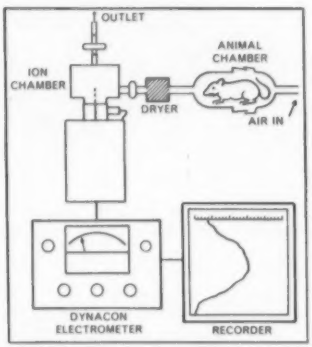


Operator places 250 ml. ion chamber on Dynacon converter. Sample may be read directly in microcuries or dis./sec. on meter and recorder.



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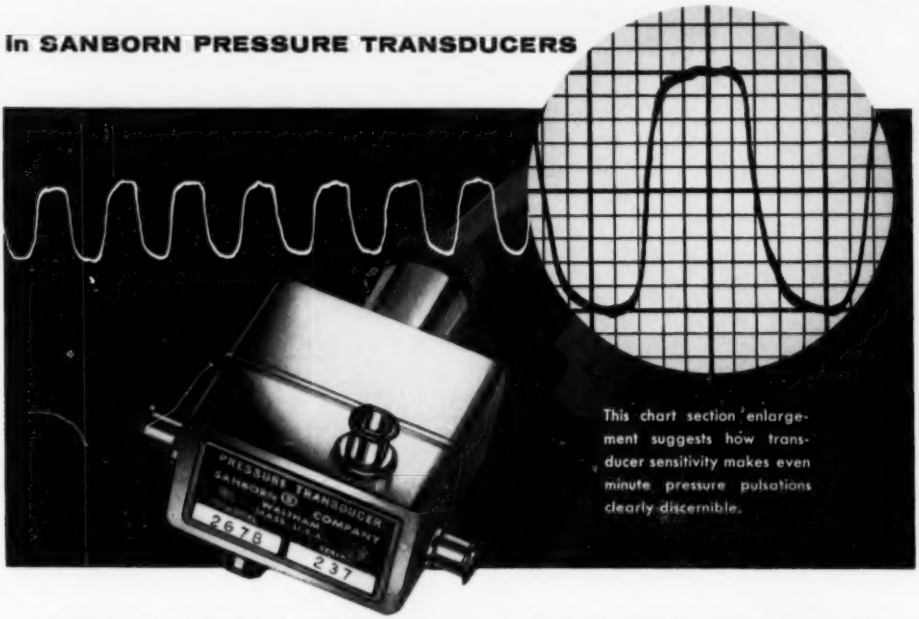

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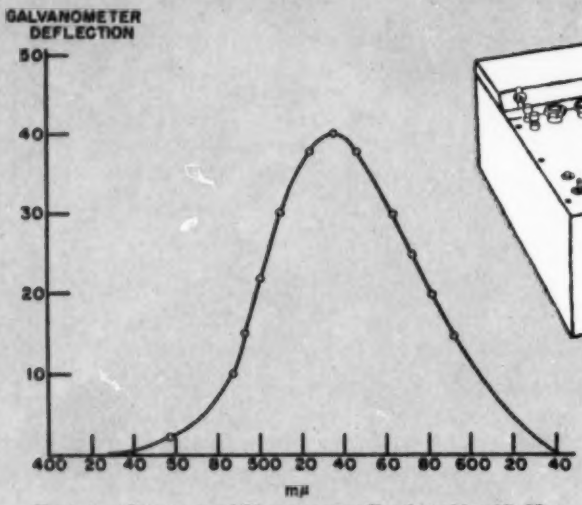

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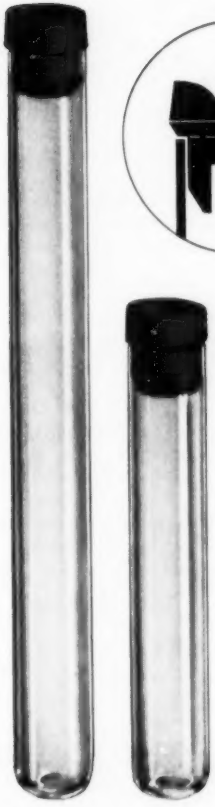

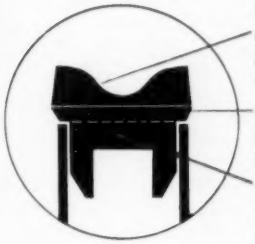

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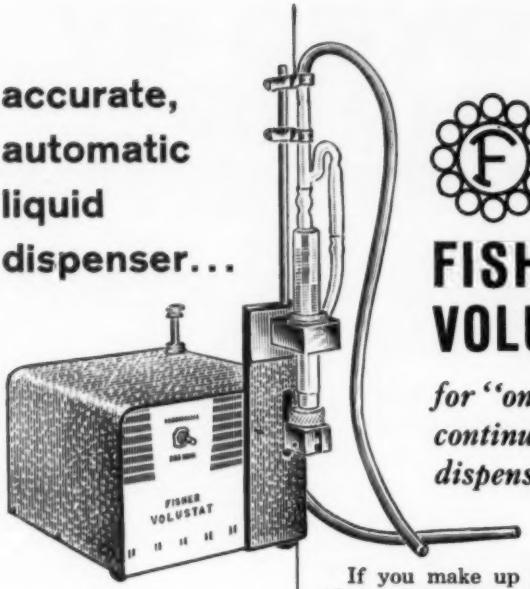

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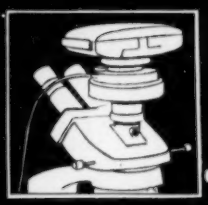

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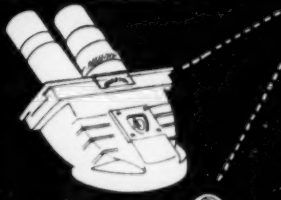

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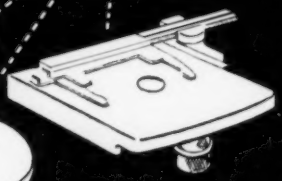

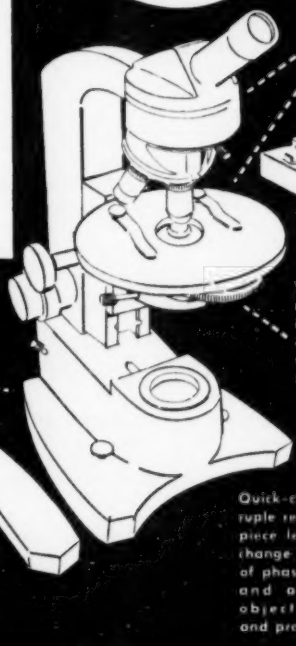

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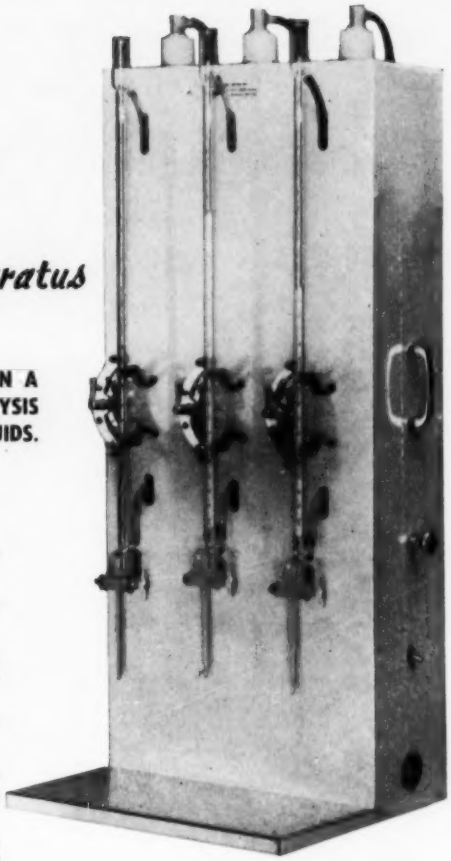

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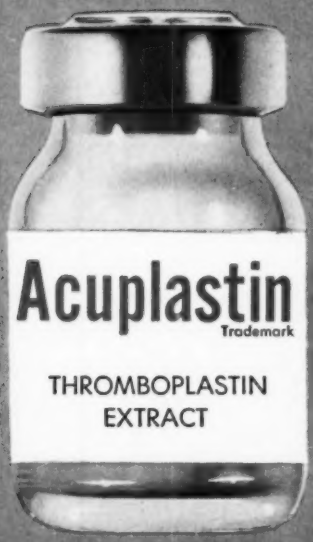

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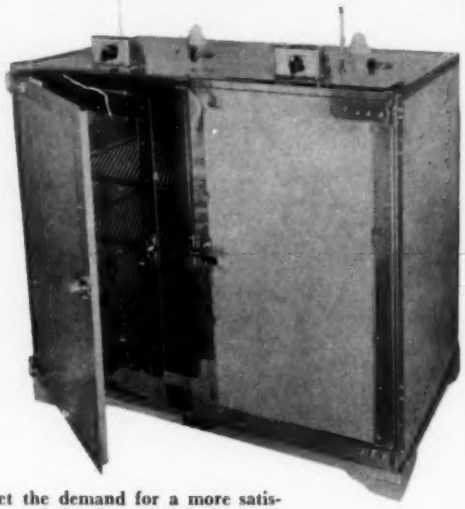

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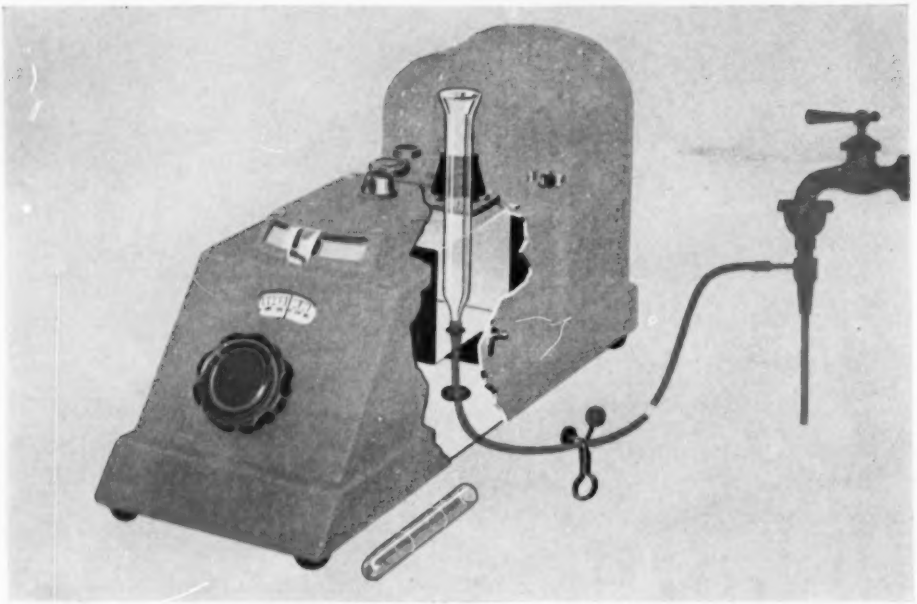

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Inside diameter approximately 12 mm, outside diameter approximately 14 mm, overall length 135 mm. With flared top to facilitate filling and with tapered tubulation 4 mm outside diameter at bottom for rubber tubing connection to waste receiver or aspirator.

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3788-E2. Colorimeter Tube, Drain Type, Thomas-Seligson, capacity 10 ml, as above described, with directions for use..... **1.40**
10% discount in lots of 12.

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A suitable waste connection can be provided by a 1 ft. length of 8848 Rubber Tubing and a 3278 Pinchcock, 2¼ inches long. Longer tubing will be required for connection to an aspirator, depending on location of faucet.

5115-V. Neoprene Adapter, size No. 3..... **.57**
8848. Rubber Tubing, 3 mm bore, 1 ft. long; for connection of Drain Tube to waste reservoir. .....**Per foot .12**
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The Journal of Laboratory and Clinical Medicine

VOL. 53 FEBRUARY, 1959 NO. 2

CLINICAL AND EXPERIMENTAL

THE FINDING OF URINARY PROTEOSE IN CERTAIN RENAL DISEASES

ISOLATION AND IDENTIFICATION

JACOB M. LEVINE, M.D., ALVIN DUBIN, M.S., AND S. HOWARD ARMSTRONG, JR., M.D.
CHICAGO, ILL.

THE most common protein precipitants employed in the routine evaluation of proteinuria are sulfosalicylic acid, trichloroacetic acid, and heat and acetic acid. While it has been known for many years that there are qualitative differences in the proteins of urines from patients with varied diseases,[1] it has not always been possible to differentiate by simple chemical means between the proteinuria of various types of active renal disease and that of congestive heart failure, pregnancy, and other extrarenal proteinurias by these precipitants or indeed by more elaborate techniques of physical chemistry.

In the routine analysis of urines from nephritic and cardiac patients, one of us (J. M. L.) noted that the initial urines of a patient with active glomerulonephritis confirmed by biopsy yielded heavy precipitates on first boiling, followed by acidification but much less to no precipitate when the order was reversed, that is, the urine first acidified with acetic acid and then boiled. (The details of clinical and laboratory findings on this patient are given in the Appendix.) The protein-containing urines from certain cardiac patients and from certain pregnant women examined at the same time did not reveal this phenomenon.

The phenomenon suggested presence of a heat-resistant, water-soluble substance, protein-like in nature.

From the Hektoen Institute for Medical Research of the Cook County Hospital and the Department of Medicine of the University of Illinois College of Medicine, Chicago, Ill.

These studies have been supported by Grant A-927 from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service.

Presented in part before the Thirty-first Annual Meeting of the Central Society for Clinical Research, Chicago, Oct. 31 and Nov. 1, 1958.

Received for publication June 16, 1958.

The usually accepted definition of proteoses is in terms of this differential solubility, ". . . soluble in water, non-coagulable by heat, and precipitated by saturating their solutions with ammonium sulfate,"[2] and carries no a priori implication as to molecular size or shape.

The purpose of this report is twofold: (1) to describe the isolation of such a proteose; its gross solubility, electrophoretic, and certain other chemical characteristics and (2) to present a preliminary survey by a simple clinical test of the incidence of this proteose in proteinurias of a variety of clinical classifications.

METHODS

Isolation of Proteose.—Proteose-containing urine was acidified with acetic acid to pH 5.2, boiled for 5 minutes, the globulins and albumins precipitated, and the urine filtered. To the filtrate was added ammonium sulfate to full saturation to assure complete salting out of the proteose.

The proteose precipitate was separated by centrifugation, the supernatant poured off, and the proteose taken up into solution again with a small amount of distilled water. The supernatants showed no further precipitation with 5 per cent sulfosalicylic acid or ammonium sulfate.

The resulting proteose was dialyzed against veronal buffer of pH 8.6, 0.1 ionic strength, at -5° C. for 3 days, changing the buffer each day, and studied electrophoretically.

Electrophoretic Studies.—Zone electrophoresis was performed with quantitation of the bromphenol blue color[3] of the paper strips with a photometric scanner (Spinco Analytrol).

Another electrophoretic strip of the same proteose was stained with periodic acid leucofuchsin (Schiff reaction) for protein-bound carbohydrate.[4]

Hexose and Hexosamine.—The protein-bound hexoses were determined according to Lustig and Langer,[5] and the protein-bound hexosamines were determined according to the method of Elson and Morgan.[6]

Clinical Test for Proteose Identification.—(1) Freshly voided urine is filtered, acidified with acetic acid to a pH between 5.0 and 6.0, brought to a boil, boiled for 5 minutes, and filtered. (2) Two milliliters of the filtrate is then layered over 2 ml. of saturated trichloroacetic acid.

The development of a ring of white precipitate at the junction of the layers constitutes a positive test for proteose. A rough quantitative estimate is arrived at by the density and height of the precipitate at the junction and is graded from 1 plus to 4 plus.

RESULTS

Properties of the Isolated Proteose.—From all the evidence to date, the substance that we have isolated from the urine of patients with nephritis is characterized by the following: (1) precipitated by sulfosalicylic and trichloroacetic acids; (2) precipitated by 0.25 M trichloroacetic acid, goes into solution when heated to 93° to 97° C., precipitating again when cooled between 90° and 70° C. (this phenomenon can be repeated a multiple number of times); (3) not coagulated by heat; (4) although not precipitated by half saturation, precipitation occurs on full saturation with ammonium sulfate; (5) nondialyzable, and not precipitated by dialysis against distilled water; (6) biuret-positive, giving a weak reaction; (7) electrophoretically characterized by its position predominantly at alpha-1, and extending almost to the alpha-2 position (Fig. 1);

(8) contains approximately 30 mg. of bound hexose and 50 mg. of bound hexosamine per 100 mg. of nitrogen; and (9) reported by Dr. R. J. Winzler as giving a strongly positive precipitin reaction with orosomucoid antisera. (This may be an instance of a cross reaction reported for proteins of high carbohydrate content.)[7]

The solubility properties displayed by the substance isolated from the urine fulfills the criteria set forth in the definition for proteoses given in the introduction.

Further physical and chemical studies are being conducted on this proteose and will be reported in a future communication.

Fig. 1.—Patient J. McQ. Strip A, serum electrophoresis with veronal buffer at pH 8.6, 0.1 ionic strength, and stained with bromphenol blue. Strips B and C, isolated proteose electrophoresis under the same conditions, but with B stained with bromphenol blue and the C with periodic acid leucofuchsin (Schiff reaction). Note correspondence of position of chief component in B and C with the chief (faster) component in C of Fig. 3.

Patients.—Patients with proteinuria were selected from the wards at Cook County Hospital, and the urines from nephritic patients who had been followed for periods up to 2 years in the Research Nephritic Clinic at Hektoen Institute were studied. The usual methods of clinical classification were supplemented by renal biopsy in 13 of the 50 patients studied.

Acute Glomerulonephritis (Acute Phase).—A detailed report of the clinical, laboratory, and renal biopsy findings is given in the appendix for Patient R. B. on whom serial studies have been made. Analysis of his urine some 10 days after sore throat and about a week after onset of edema revealed a 4+ reaction with 5 per cent sulfosalicylic acid, and a reaction of the same intensity with heat, but no precipitate formed when the urine was first acidified with acetic acid and heated to boiling. The proteose reaction was 3+.

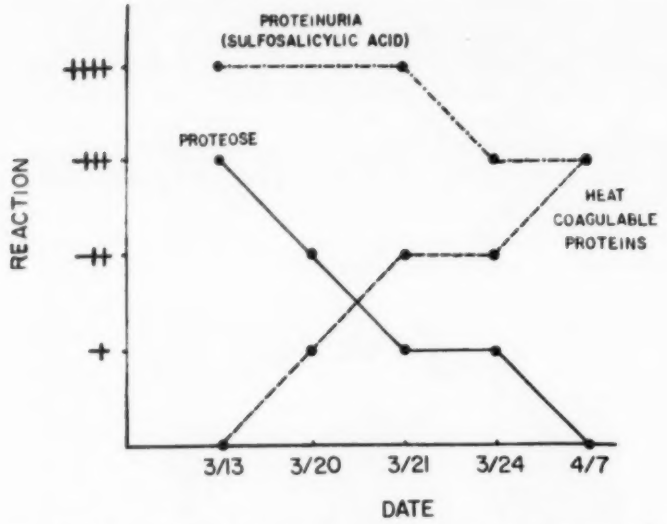

Fig. 2.—Patient R. B. For identification of dates relative to clinical course, sore throat appeared about 3/4 and generalized edema appeared about 3/8, was almost gone about 3/24, and completely gone 4/7 (see text and Appendix).

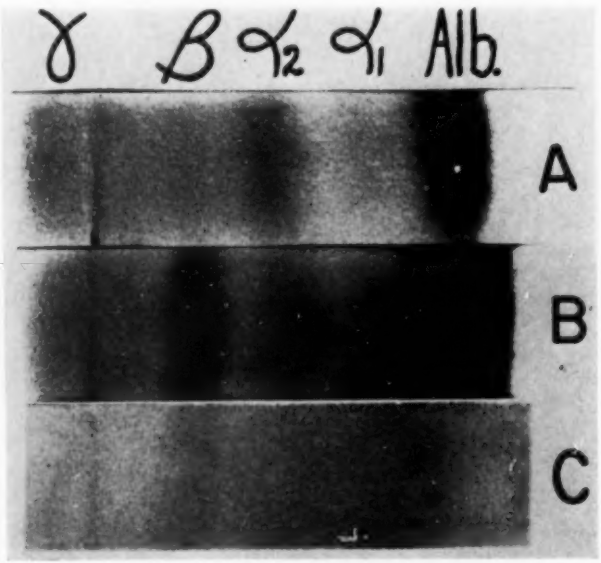

Fig. 3.—Patient R. B. Strips A and B, serum and urine electrophoresis, respectively, with veronal buffer at pH 8.6, 0.1 ionic strength, and stained with bromphenol blue. Strip C, urine electrophoresis under the same conditions but stained with periodic acid leucofuchsin (Schiff reaction). Compare C with B and C of Fig. 1.

One week later, when edema was almost gone, the urine still gave a 4+ reaction with sulfosalicylic acid, but a precipitate now appeared when the acidified urine was heated to boiling. The proteose reaction was less, about 2+. Subsequent analyses revealed that the detectable heat-coagulable proteins increased while the proteose content decreased until about a month after onset (with patient asymptomatic and edema free) there were only heat-coagulable proteins with no proteose to be detected. These findings are illustrated in Fig. 2.

Serum and urine protein electrophoreses were performed by methods previously described from this laboratory,* and the patterns of the urine disclosed a trailing of the albumin corresponding to the alpha-1 position. An electrophoretic strip of urine collected some 2 weeks after onset of edema, when treated with the periodic acid-Schiff reagent, revealed a positive reaction at the alpha-1 position (Fig. 3).

TABLE I. HEALED ACUTE GLOMERULONEPHRITIS

PATIENT	ORIGINAL DIAGNOSIS	URINE STUDY			
		TIME AFTER ACUTE EPISODE (MONTHS)	SULFOSALICYLIC ACID	ACETIC ACID AND HEAT	PROTEOSE
M. M.*	Biopsy	8	0	0	0
E. G.†	Biopsy	12	0	0	0
P. Sn.‡	Biopsy	11	0	0	0
R. G.§	Clinical	17	0	0	0
M. P.§	Biopsy	5	0	±	0
A. W.‖	Biopsy	17	+ + +	+ + +	0

*Repeat biopsy at time of the above urine study showed glomerular healing, but also a persistent interstitial nephritis which, in certain areas, was severe enough to be associated with tubular atrophy and as many as 4,000 colonies of mixed flora of gram-positive and gram-negative organisms per milliliter of catheterized urine. (It is of interest that despite initial recovery of Type 12 beta hemolytic streptococci, the antistreptolysin and antistreptokinase titers never rose above normal limits.)

†This patient's antistreptolysin maximum was 833 with rapid subsidence after acute episode. While some 6 months after this episode there was transitory suspicious evidence of a superimposed pyelonephritis (by physical examination and examination of urine sediment and bacteriology), at the time of the study of urine here reported this had subsided under therapy.

‡Renal biopsy 2 months after acute episode showed, in addition to hypercellularity of glomeruli and an occasional inflammatory cell, some early lobulation and adhesions. Repeat biopsy 4 months after acute episode showed regression of the former glomerular changes but persistence of the latter at a time when the patient, by ordinary clinical criteria, was well, and urine showed only evanescent proteinuria and an occasional cast. At the time of urine study here reported, these had disappeared.

§Multiple urine examinations subsequent to this study disclosed complete absence of protein and pathologic-formed elements.

‖Type 12 beta hemolytic streptococci were recovered at the time of acute episode. This patient's antistreptolysin maximum was 330 at that time and has fluctuated around the 200 level to date. While proteinuria has persisted, it is an intermittent finding in monthly examinations. Red cells have been absent from sediment for at least 6 months prior to the urine examination here reported; the only remaining pathologic-formed elements are limited to sparse granular casts.

Acute Glomerulonephritis (Apparent Clinical Recovery).—In Table I are given urine protein and proteose studies for 6 patients whose hemorrhagic glomerulonephritic episodes occurred over 5 months previously. Diagnosis was confirmed by renal biopsy in 5. (For details of 2 follow-up renal biopsies, see footnotes to Table I.) In the urines of all 6 patients no proteose was found; in 4 proteinuria had also disappeared and in 2 patients transient proteinuria persisted despite absence of hematuria, clinical well-being, and creatinine clearances which, in serial follow-up studies, stabilized close to normal limits.

Chronic Glomerulonephritis (Progressive Course).—Spot checks were made on the urines of 16 patients with chronic glomerulonephritis. In 5 of these cases the diagnosis rests on renal biopsy and in the remainder on the usual clinical criteria. Of this group, one adult was in a hematuric edematous exacerbation, and 4 were in a chronic nephrotic state (3 of the latter were children). Six adults had progressed to a stage of chronic nitrogen retention of serious degree. Of the remainder, all but 2 showed creatinine clearances well below the normal range. Six adults showed evidence in the formed elements of urine as well as by culture of superimposed pyelonephritis of varying degree.*

A positive test for proteose was found in each of this heterogeneous group of chronic glomerulonephritics. In no instance, was the proteose found in the absence of simultaneous proteinuria although there was a considerable variation noted between the qualitative degree of proteinuria and proteosuria.

While, at this stage of the study, we have failed to find suggestive correlations between degree of proteosuria and apparent rate of progression of the renal lesion in individual patients, the universal proteosuria of this group stands in contrast to the acute glomerulonephritic patients of the preceding section (in whom histologic evidence of healing of the inflammatory glomerular changes is associated with absence of proteosuria).

Pyelonephritis Associated With Obstructive Uropathy.—In a study of the urines from 5 patients whose diagnoses rested on obvious clinical grounds, bladder urines alone were used in 3; in the fourth, simultaneous study of bladder urine and urine from a nephrostomy drainage was carried out; in the fifth, simultaneous study of bladder urine and urine from both kidneys on ureteral catheterization was carried out. None of these patients were diabetics.

In all urines studied, some degree of proteosuria accompanied clear-cut proteinuria.

Nephropathies Associated With Diabetes.—The urines of 6 patients with nephropathy associated with diabetes mellitus were studied.

In one of these, the diagnosis of intercapillary glomerulosclerosis was made by biopsy, and superimposed pyelonephritis was suspected because of polymorphonuclear leukocytic casts and bacteriologic evidence. In 3 others, a diagnosis of intercapillary glomerulosclerosis was inferred on the basis of diminished serum albumins in the presence of massive proteinuria. In one of these, evidence of superimposed pyelonephritis was also found; 2 showed no evidence of nitrogen retention; 3 showed minimal nitrogen retention; one was in fatal uremia.

All showed proteosuria of some degree associated with the proteinuria. There was no clear-cut parallelism between the degree of proteinuria and proteosuria as studied by these qualitative methods.

Proteinurias the Origin of Which Is Presumably Not Associated With Inflammatory or Exudative Renal Changes.—The chronic glomerulonephritics, obstructive uropathies, and diabetic nephropathies of the above 3 sections stand

*Detailed clinical courses of these patients together with serial electrophoretic analysis of urine proteins are in preparation in collaboration with Dr. D. S. Kushner.

in contrast to a group of 10 patients who, though comparable in degree of qualitative proteinuria, showed no evidence of proteose in the urine. Of these, 8 were in congestive heart failure (2 were classified as cor pulmonale, 2 as arteriosclerotic heart disease, and 4 as hypertensive heart disease). The 2 other patients in this group carried the diagnoses of metastatic carcinoma and chronic relapsing pancreatitis, respectively.

Miscellaneous Proteinurias.—One patient with a clinical diagnosis of lupus erythematosus (whose renal biopsy showed glomerular changes consistent therewith) showed proteosuria in association with the proteinuria. Two patients with the same diagnosis and in the absence of evident renal involvement showed no proteosuria or proteinuria. In addition, 3 patients with multiple myeloma were studied; 2 showed evidence of Bence Jones proteinuria, and one showed proteinuria in the absence of Bence Jones proteinuria. All 3 patients showed positive qualitative tests for proteosuria. The proteose findings may be explained by the presence of Bence Jones protein which is proteose in nature.[9]

DISCUSSION

The literature is replete with investigations of proteinuria, and innumerable studies have been conducted on sera and urines of nephritic patients.[10-15] The problem of the nature of the nephritic proteinuria has been approached essentially from these directions: (1) the qualitative whereby the stability of a gold sol was studied with the urines from patients with various pathologic conditions,[1] and the specific serologic reactions whereby sera drawn during the edematous stage of nephrosis were reacted with antibodies from rabbits hyperimmunized with serum globulin from nephritic patients[16]; (2) the quantitative whereby proteins were fractioned into albumins and globulins, studying their ratios and comparing the findings with that of sera.[11, 12] Most recently, electrophoresis by the paper strip method has been employed for the separation and quantitation of serum and urinary proteins in nephritis.[15]

By the usual methods for urinary protein precipitation, such as sulfosalicylic acid or trichloroacetic acid, the proteose also precipitates. By these techniques, it was impossible to differentiate the proteose from the albumin and globulin. These techniques overlooked the possibility of the presence in urine from nephritic patients of a heat-soluble protein which is precipitated with sulfosalicylic acid and trichloroacetic acid, but is released from the precipitate on boiling and then recovered in the filtrate[9]: a protein which is left in solution after the coagulation of albumin and globulins by acidifying and boiling the urine. These were the properties which led us to the isolation of the proteose.

Beckman and associates[17] reported the isolation of a protein soluble in trichloroacetic acid. No mention of the etiology of the proteinuria is made. However, a comparison of the properties of their protein with the proteose isolated by us from the urine of nephritic patients does not reveal identity. While the Beckman protein was not precipitated by 0.25 M trichloroacetic acid, the material here reported is so precipitated. The Beckman protein was precipitated by heat and acetic acid while the proteose reported here is soluble, and

this is the basis for the separation of the proteose in our proposed diagnostic test for active renal disease. While the proteose is not precipitated until full saturation with ammonium sulfate, the protein of Beckman and associates was precipitated by half-saturated ammonium sulfate. Electrophoretically, the mobility was similar. The authors do not report whether their protein contained carbohydrate. The proteose reported here gives a strongly positive test for carbohydrate with the periodic acid-Schiff reagent (Fig. 1).

Popenoe[18] reported an investigation of the trichloroacetic acid-soluble protein of urine reported by Beckman and co-workers[17] and concluded (1) that the principal acid-soluble protein of children with nephrotic syndrome is indistinguishable from the alpha-1 acid glycoprotein isolated by Schmid[19] from normal human plasma by electrophoresis, by analysis for hexose, hexosamine, and nitrogen, and by a specific precipitin test and (2) that it is not unique to pathologic urine. The glycoprotein reported by Popenoe[18] did not form a precipitate on heating in dilute acid or on half-saturation with ammonium sulfate and was soluble in 0.25 M trichloroacetic acid, 1.8 M perchloric acid, and in 0.6 M sulfosalicylic acid fulfilling the criteria for the purified acid glycoprotein of normal plasma.[19, 20] The proteose isolated by us from the urine of nephritic patients is precipitated by all the above-mentioned acids of the stated concentrations. The comparison of the properties of the materials reported by Beckman and associates,[17] Popenoe,[18] and of the proteose reported here is summarized in Table II.

TABLE II

REAGENTS	BECKMAN AND ASSOCIATES[17]	POPENOE[18]	MATERIAL HERE REPORTED
0.25 M trichloroacetic acid	Soluble	Soluble	Precipitated
Acetic acid and heat	Precipitated	Soluble	Soluble
Salting out with ammonium sulfate	Half saturation	Full saturation	Full saturation
1.8 M perchloric acid		Soluble	Precipitated
0.6 M sulfosalicylic		Soluble	Precipitated

In a most recent and extensive study of the protein-bound carbohydrates in human serum and urine in proteinuria, Laurent[21] reported that in pathologic sera a wide range of quotient values was found indicating the presence of abnormal proteins and/or pathologically increased amounts of normally occurring protein fractions with carbohydrate quotients deviating from the average values. In proteinuria, the albumins and globulins of the urine contained hexose in approximately the same amounts as the serum. There were no salient differences between the bound-carbohydrate quotients of the sera investigated and between the corresponding serum and urine fractions. The results from his study of 13 normal human sera, 86 sera obtained from patients with kidney disorders and miscellaneous conditions, and 44 urine samples from patients with proteinuria of varied origin led Laurent to the conclusion that no loss of bound-carbohydrates occurred during the passage of protein through the kidneys.

Goettsch and Lyttle[16] reported in their precipitin studies in nephrosis and nephritis that the altered protein in the globulin fraction of 3 patients, when

injected into the rabbit, formed antibodies which did not react with normal globulin; the antibodies reacted with nephrotic sera drawn during the edematous stage of nephrosis but not with the sera of healed nephrotic patients. These temporal relations are analogous, although not identical, with the sequences noted above for urinary proteose in the serial study of Patient R. B. (acute glomerulonephritis). We have no comparable sequential studies in our chronic glomerulonephritic group; in part, because it is chiefly adult, and "healing" in an adult glomerulonephritic nephrotic patient is rare.*

Our preliminary studies lead us to believe that the occurrence of urinary proteose (in contradistinction to protein), may be correlated with active parenchymal renal inflammatory changes. Proteosuria per se does not yet, in our experience, distinguish between glomeruli and tubules as primary foci of inflammation or between direct bacterial and autoimmune pathogenesis. The absence of proteosuria in our proteinurias whose pathogenesis is probably not correlated with parenchymal inflammation leads us, in rather old-fashioned terminology, to the suspicion that, while proteinuria may be transudative or exudative, proteosuria is clinically indicative of an exudative process.

SUMMARY

1. The isolation of a proteose from the urine of nephritic patients is reported. This proteose is electrophoretically characterized by its position predominantly at alpha-1, extending almost to the alpha-2 position, and yields a strongly positive carbohydrate reaction with the periodic acid-Schiff reagent.

2. A simple test based on the solubility of this proteose is presented, with evidence (based on a study of 50 patients in diagnostic categories including acute active and "healed" glomerulonephritis, chronic glomerulonephritis, pyelonephritis, diabetic nephropathy, and congestive heart failure) suggesting its utility in the clinical differentation of active parenchymal renal inflammation from proteinurias produced by other mechanisms.

APPENDIX

R. B. (C. C. H. 58-17226), an 18-year-old Puerto Rican male, was admitted with chief complaint of periorbital edema of 4 days' duration. One week before admission, he had a sore throat which had subsided by the time of edema appearance. He denied visible hematuria, headache, prior episodes of edema, or any other phenomena suggestive of glomerulonephritis or pyelonephritis.

On physical examination, the only significant abnormal finding was a minimal generalized puffiness noted particularly in the face, hands, sacrum, and ankles. Specifically, blood pressure was within normal limits as were the fundi and heart size.

The initial opinion of the renal team favored acute glomerulonephritis as a clinical diagnosis; however, in the absence of gross hematuria, one member believed the proper classification to be an exacerbation of a chronic membranous glomerulonephritis.

Although his initial urine was not grossly bloody, on microscopic examination it was loaded with red cells and, in addition, contained a few white cells and granular and hyaline casts in the centrifuged sediment. Neither white nor red cell casts were noted in the first 2 weeks of his course, and there were no white cell clumps.

*Ongoing serial studies in collaboration with Dr. Aaron Grossman of Cook County Children's Hospital are aimed at elucidation of this point.

His initial serum albumins were 3.6 Gm. per cent, total globulins 1.5 Gm. per cent, gamma globulins 1.1 Gm. per cent, and cholesterol 147 mg. per cent.

An initial nonprotein nitrogen of 70 mg. per cent had returned to 32 mg. per cent at the end of the first week of hospitalization and his edema had disappeared. At this time, his 24-hour proteinuria amounted to 9.1 Gm.

At the end of his second hospitalized week, proteinuria amounted to 6.1 Gm. per 24 hours, creatinine clearance was 86 ml. per minute, serum creatinine 1.0 mg. per cent, total serum lipids were 580 mg. per cent, lipid phosphorus 9 mg. per cent, serum mucoprotein was 81 mg. per cent, and gamma globulins were 0.85 Gm. per cent.

His initial urine culture showed no growth. His initial throat culture showed no beta hemolytic streptococci. However, while his initial antistreptolysin titer taken at the end of 2 weeks was 250, by the fourth week it had arisen to 333. The corresponding antistreptokinase values were 160 and 320, respectively. By the sixth week, the antistreptolysin titer had fallen to 250, and it remained at this level or below for the remainder of his course. His antistreptokinase titer, which was 160 2 weeks after admission, rose to 320 4 weeks after admission and thereafter returned to its initial normal level.

Renal biopsy, carried out one month after admission, was consistent with a diagnosis of proliferative glomerulonephritis, with findings suggestive of a chronic membranous process at a minimum. (Detailed report of histology is in preparation for publication in collaboration with Dr. P. B. Szanto.) Culture of this biopsy was sterile.

At this time, multiple urine examinations continued to show 4+ proteinuria; 24-hour proteinuria amounted to 3.4 Gm. Microscopic hematuria had all but disappeared and the formed elements of the sediment were limited to a few white cells and hyaline casts.

When seen up to 3 months after admission in the Research Renal Clinic, he continued to be asymptomatic and normotensive with normal serum cholesterol, lipid phosphorus, total lipids, and a creatinine clearance remaining in the 90's. However, a 1+ to 2+ proteinuria and minimal microscopic hematuria has persisted. On one occasion, white cell clumps were seen in the urine. Two urine cultures were sterile; a third showed nonhemolytic staphylococci (albus) with a colony count of only 440 per milliliter of urine.

We are indebted to Warden Fred Hertwig, Cook County Hospital, and to Drs. Leonard H. and Louis D. Weissman, Medical Research Foundation, for the provision of basic facilities for protein research at the Hektoen Institute.

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EFFECTS OF IONIC STRENGTH ON THE RELATIVE MOBILITY OF ABNORMAL SERUM PROTEINS

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ABNORMAL serum globulins, such as myeloma globulins, macroglobulins, or cryoglobulins, are usually characterized, electrophoretically, by a high narrow peak, corresponding to a relatively large concentration and a high degree of electric homogeneity.[1] The position of the peak may lie with the γ-globulins, between the γ- and β-globulins, with β-globulins, or more rarely with the α-globulins, and a plot of accumulated data on myeloma protein mobilities at pH 8.6 gives a bimodal distribution curve with one maximum in the gamma region and a second maximum in the β-globulin region.[2] Although myeloma globulins whose mobilities correspond to normal γ-globulins usually have S_{20} and D_{20} values in the normal γ-range, the individuality of each abnormal globulin, i.e., patient-specificity, is emphasized by immunologic investigations.[3]

Previous electrophoretic studies of normal serum proteins have shown that the relative mobility of various serum globulins may be altered grossly by variations in ionic strength of the buffer.[4] Since this alteration appears related to such factors as axial ratio of the molecules and specific ion binding, it was felt that an investigation of the dependence of mobility upon ionic strength would provide a further comparison of abnormal to normal globulins. Consequently 5 different human sera, 1 normal and 4 containing abnormal globulins, were subjected to electrophoresis simultaneously, at a number of different buffer concentrations. The sera include a myeloma protein with the mobility of γ-globulin, one with the mobility of β-globulin, and 2 macroglobulins. The results are reported below.

MATERIALS AND METHODS

Patients.—The serum employed as a normal control was obtained from a 23-year-old woman, apparently in good health.

Serum A was from a patient who had multiple myeloma, with an abnormal protein whose electrophoretic mobility corresponds to γ-globulin. The clinical diagnosis was confirmed by autopsy.

Serum B was from another patient with multiple myeloma and an abnormal protein whose electrophoretic mobility corresponds to β-globulin. The clinical diagnosis was confirmed by autopsy.

Serum C contained an abnormal protein shown by ultracentrifugal analysis to be macroglobulin. There were 3 components with sedimentation constants of 4.58, 178, and 23.68.* Water dilution and temperature viscosity index were also positive. In addition, the serum

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*The ultracentrifugal analyses and sedimentation constant calculations were carried out by Dr. H. Ko of The Upjohn Company, Kalamazoo, Mich.

contained a pyroglobulin.[5] The macroglobulin is included in the pyroglobulin fraction, since electrophoresis of the serum, after removal of pyroglobulin, no longer showed the presence of macroglobulin.

Serum D contained a macroglobulin, diagnosed on the basis of water dilution, temperature viscosity index, and ultracentrifugal analysis. The latter again indicated 3 components, with sedimentation constants of 4.5S, 17S, and 23.6S.*

Electrophoresis.—The electrophoresis was conducted by the fluid film method, which has been described previously.[6] A veronal buffer of pH 8.6 was used at the following concentrations: 0.01 M, 0.02 M, 0.033 M, 0.066 M, 0. 1M, and 0.15 M. At each concentration, all 5 sera were migrating simultaneously in the same buffer solution, thus tending to cancel out possible variations that could affect the comparison of different sera at the same ionic strength. The electrophoresis was conducted for a period of 4½ hours at 160 volts, corresponding to a potential gradient of 5.0 volts per centimeter.

RESULTS

The electropherograms of the 5 sera, at each of the 6 buffer concentrations, are shown in Fig. 1. Densitometer curves (optical density of the stained electropherogram along the migration path) for the normal controls were obtained with a Kelab densitometer and are presented in Fig. 2. The relative mobility of the 4 abnormal globulins was determined from the position at which the outside edge of the migrating band was parallel to the direction of the electric field. Each abnormal globulin is designated in Fig. 2 by a bar indicating its position when the normal and abnormal electropherograms are superimposed.

It is seen that mobility varies with buffer ion concentrations. For example, as the buffer concentration decreases, globulin A moves progressively closer to the rear or toward the cathode in the same electropherograms and also as compared to the γ-globulins of the normal control, Fig. 2. The mobility of globulin B, however, compared to the mobility of normal β_1-globulin appears constant. The macroglobulins remain at the point of origin in the 0.01 M and 0.02 M buffers. In the 0.033 M buffer, macroglobulin C begins to migrate from the origin, and a new faint band appears toward the cathode, suggesting an equilibrium between soluble and relatively insoluble fractions of this protein. Macroglobulin D does not migrate until the buffer concentration reaches a value of 0.066 M. The 6 normal electropherograms, corresponding to the curves of Fig. 2, are reproduced schematically in Fig. 3. The scales of the abscissa are altered as above.

Since each macroglobulin does not migrate from the origin until the ionic strength reaches a specific value, the absorbance of sera C and D, each diluted 1 to 50 with veronal buffer, was measured at various buffer ion concentrations in a Coleman Universal spectrophotometer, 580 mμ. As Fig. 4 shows, an increase in turbidity, which can be correlated with an increasing particle size due to aggregation or decreasing solubility, occurs as the buffer concentration decreases. The concentration at which the absorbance begins to increase is slightly below 0.04 M for serum C, and below 0.07 M for serum D. These values correspond to the respective buffer concentrations at which the 2 macroglobulins begin to migrate from the origin.

*See footnote on opposite page.

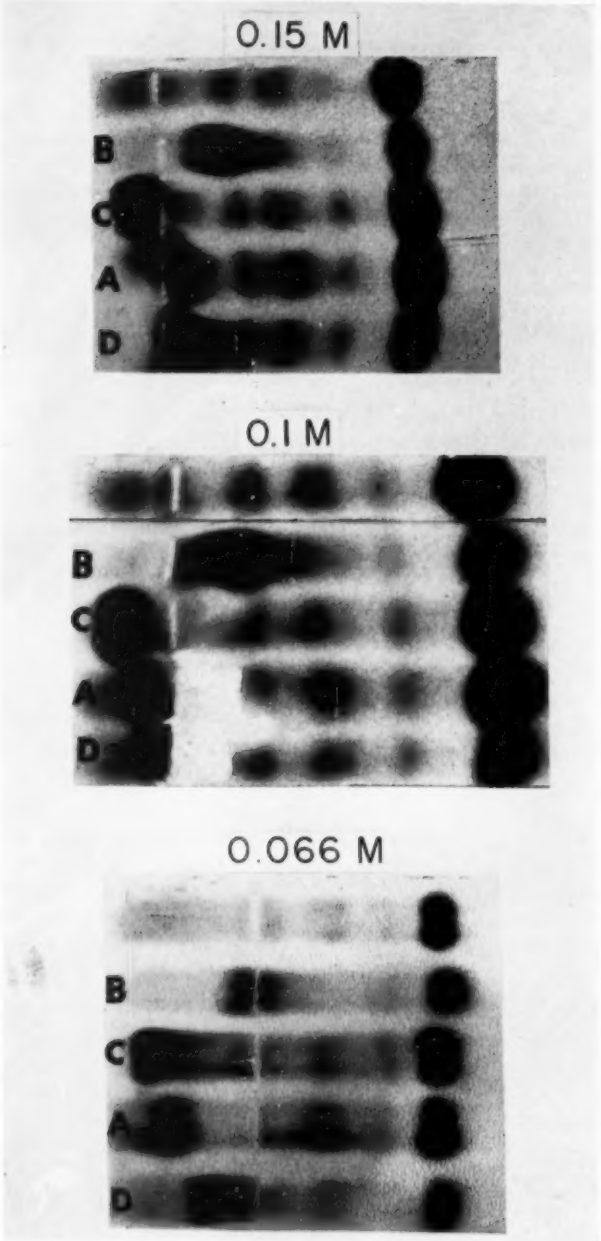

Fig. 1A.

Figs. 1A and 1B.—Electropherograms at 6 indicated veronal buffer concentrations. In each case, the top serum is the normal control, while the other sera are referred to in the text by the designated letter.

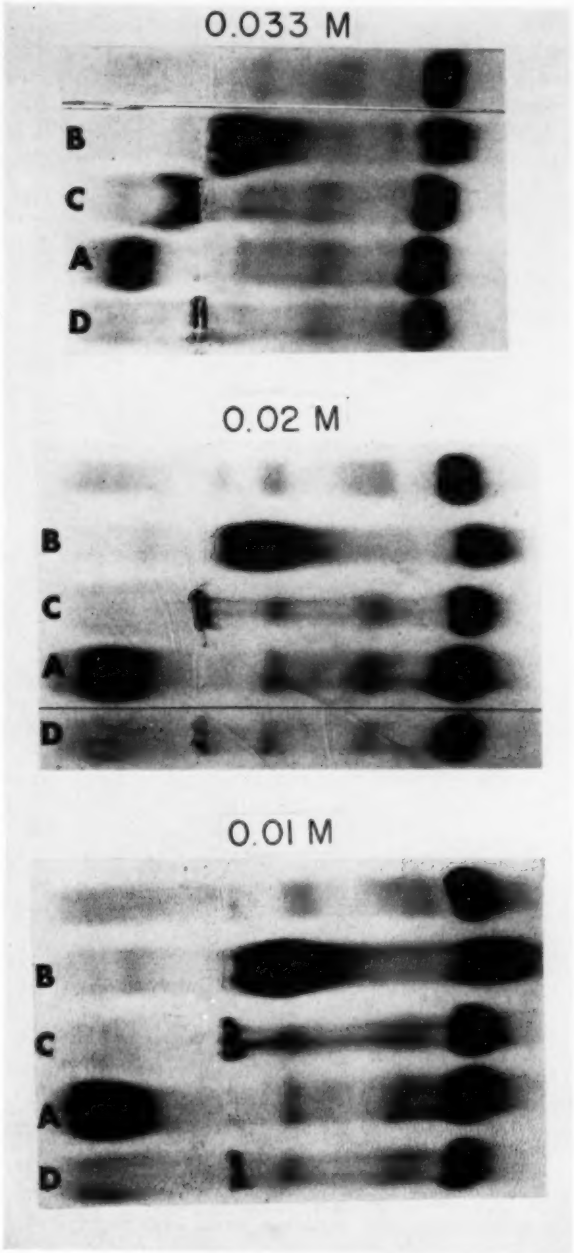

Fig. 1B.—*(For legend see opposite page.)*

DISCUSSION

The dependence of relative mobility of normal serum proteins upon ionic strength agrees with results obtained previously.[4] With a decreasing ionic strength, the results can be summarized as follows: (1) the relative mobility of α_1-globulin increases until, at a veronal concentration of 0.01 M, it becomes incorporated into the albumin band; (2) the relative mobility of the α_2-globulin also increases; (3) the width of the α_2-globulin band increases suggesting a dissociation into further components; (4) the β-globulin dissociates into a β_1- and a β_2-component. γ_1- and γ_2-globulin can be seen as distinct fractions only in the two highest buffer concentrations.

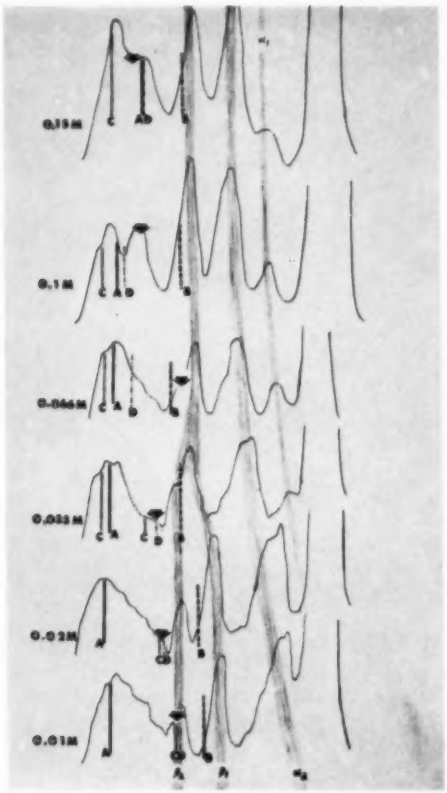

Fig. 2.—Densitometer curves of the normal control of Fig. 1. Relative mobilities of the abnormal globulin of each serum are indicated.

The alteration in the relative mobility and increasing separation of the various protein components with variation in ionic strength may be related to both the retarding effect of the ionic atmosphere and to binding effects between

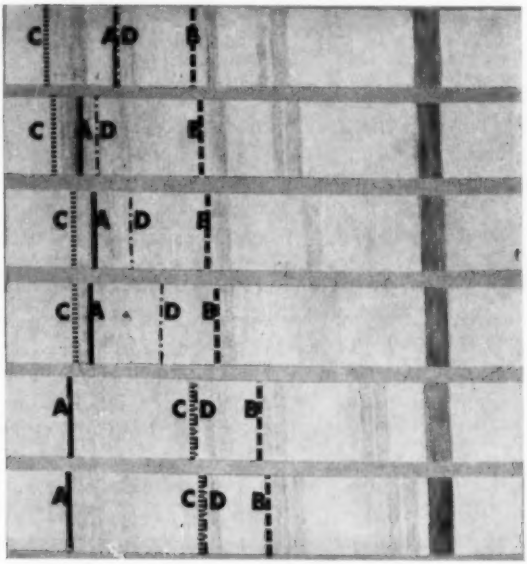

Fig. 3.—Electropherograms corresponding to the curves of Fig. 2, reproduced schematically to indicate the relative positions of the abnormal globulins.

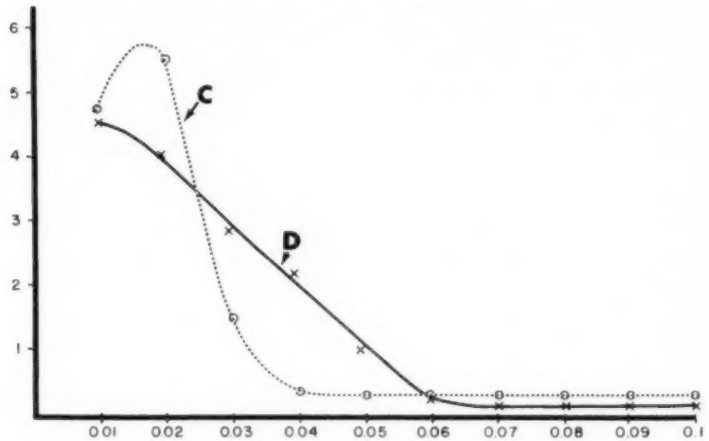

Fig. 4.—The absorbance of the 2 "macroglobulin-containing sera" after a fiftyfold dilution with buffers of varying concentration. Ordinate is absorbance and abscissa is buffer concentration. The drop in the absorbance of serum C in most dilute buffer was due to the formation of a heavy precipitate.

the proteins and buffer ions. The mobility (μ) of a protein molecule has been shown to be related to its charge (Q) by the expression[7]:

$$\mu = \left(\frac{Q}{6\pi\eta r}\right)\left(f\ (\kappa, r)\right)\left(\frac{1 + \kappa r_i}{1 + \kappa r + \kappa r_i}\right)\left(C\right)\left(f\ (\kappa, {}^{a}/_{b})\right)$$

where η is the viscosity; r is the effective protein spherical radius; r_i the average radius of the buffer ion; f (κ, r) a function correcting for field distortion by a large nonconducting particle; κ is the Debye function; C is a dimensional constant; and f (κ, a/b) is a function correcting for the asymmetry of a protein of axial ratio a/b.

It has been shown that the retarding effect of the ionic atmosphere due to the asymmetry function (κ, a/b) is quite marked and that its effect is not constant for a given shape, but increases with the ionic strength.[7] Since the serum proteins vary in regard to symmetry and axial ratio, their relative mobilities will thus be affected. A further cause for alteration of relative mobility with changing ionic strength may be unequal binding of buffer ions by the different proteins, which would thus influence the net charge, Q. Binding of buffer ions has been shown to be extensive, and to vary quantitatively, with individual proteins.[8-11]

Similar considerations would also apply to abnormal globulins. The relative shift in position of myeloma globulin A (Fig. 3) toward the rear of the gamma as the ionic strength is decreased illustrates that a myeloma globulin can exhibit the mobility of an "M" or a "gamma" type at one ionic strength, and the mobility of a "slow" type protein at another ionic strength.

The electropherograms obtained with the 0.15 M, 0.1 M, and 0.066 M buffers reveal an opposite effect of ionic strength on globulins A and D, A moving backward and C tending to move forward as the ionic strength decreases. This behavior is not exhibited by any normal protein visible in the same areas, which suggests that the abnormal globulins A and C differ, electrophoretically, from normal components. The possibility is not excluded, however, that corresponding normal components may exist, but in too low a concentration to be detected.

The macroglobulins, C and D, are the most grossly affected by ionic strength. This is due to aggregation or insolubility at lower buffer concentrations. The buffer concentrations at which the diluted serum begins to become more turbid (Fig. 4) corresponds to the buffer concentration at which each macroglobulin will no longer migrate from the point of origin (Fig. 1). The macroglobulin C shows an equilibrium between soluble migrating and insoluble nonmigrating fractions in the 0.033 M buffer. Although both macroglobulins are identical on the basis of ultracentrifugal analysis, the electrophoretic and solubility characteristics of each one are different, agreeing with the concept of "patient-specificity."[3] The heat sensitivity of globulin C is a further point of individual difference. The fact that the veronal buffer concentration at which macroglobulin D is sufficiently soluble to freely migrate lies close to 0.07 M, is especially important since paper electrophoresis is commonly conducted at a concentration of 0.05 M. Under such circumstances, the mobility of a macroglobulin may be altered by incomplete solubility. The concentration of buffer

suitable for the electrophoresis of a given macroglobulin, without modification of mobility due to incomplete solubility, can readily be determined by the method described above.

SUMMARY

Sera containing abnormal globulins have been subjected to electrophoresis in buffers varying in ionic strength. It has been found that the mobility of an abnormal globulin, compared to the normal globulins of the same serum, may change with concentrations of buffer ions. Possible reasons for this dependence are discussed.

The two macroglobulins investigated did not migrate far from the origin in buffer concentrations below a critical value, specific for each protein. This effect was found to be correlated with aggregation or insolubility of protein molecules. A simple method is described to determine what buffer concentration may be suitable for the electrophoresis of any particular macroglobulin.

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ALBUMIN METABOLISM IN NEPHROTIC ADULTS

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URINARY loss of albumin is a cardinal manifestation of the nephrotic syndrome.[1] The degree of hypoalbuminemia, however, has occasionlly seemed out of proportion to the albuminuria.[2] Recent studies have clarified the mechanism of the severe deficiency of serum albumin which may occur in nephrotic children. Using I^{131}-labeled serum proteins as tracers and immunochemical methods of assay, Gitlin and associates[3] have reported that in children with the nephrotic syndrome an abnormally increased fractional rate of catabolism of albumin, gamma globulin, and iron-binding globulin may accompany the urinary loss of these proteins and, thus, significantly contribute to their serum deficiencies.

The present report is a similar study of nephrotic adults to determine whether an abnormality of albumin metabolism occurred in the adult as well as in the childhood type of nephrosis.

METHODS

Three adult patients with latent nephrosis and 3 with overt nephrosis were studied. A summary of these patients is given in Table I.

Each patient was given a single intravenous injection of I^{131}-labeled human serum albumin consisting of 0.5 to 1.5 μc per kilogram and a total of 1 to 5 mg. of albumin. The labeled preparations were obtained from Abbott Laboratories and contained less than 1 per cent nonprotein I^{131}. These lots were also used for tracer and blood volume studies in other individuals without proteinuria and had half-lives of 12 to 17 days with less than 1 per cent I^{131} urinary excretion in 4 hours. All dilutions of the stock I^{131}-labeled albumin solution were made with saline containing 0.5 per cent human serum albumin to serve as carrier and to reduce the probability of radiation effects on the labeled molecules. Lugol's solution, 10 drops 3 times a day, was given continuously starting 48 hours before the injection of the I^{131}-labeled albumin to block thyroid uptake of I^{131}.

Complete daily urine collections were obtained on all patients. Venous blood samples were drawn every one to two days without anticoagulant and the serum separated from the clot. For the purposes of this study, plasma and serum are used interchangeably. Total I^{131} activity was measured on 3 ml. aliquots of urine and serum. Nonprotein I^{131} in the urine was determined by precipitation of the protein in a 3 ml. aliquot with 3 ml. 20 per cent trichloroacetic acid for 24 hours at 5° C. and assay of I^{131} activity in 3 ml. of the supernatant after centrifugation. I^{131} albumin activity in the urine was calculated by subtracting the nonprotein from the total I^{131} activity. The same volume of I^{131} albumin solution in the same syringe used for the injection for each patient was diluted 1:5,000 with saline containing

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0.5 per cent albumin as carrier. A 3 ml. aliquot of this dilution was counted along with all samples as a standard to refer all calculations to the administered dose. The use of this reference standard obviated the need to correct for I^{131} decay.

Plasma volume was determined by the isotope dilution method, dividing the total activity injected by the activity in the serum 12 minutes after the injection. The activity in the urine at this time was less than 0.1 per cent and could be neglected. The 12-minute interval was selected as allowing adequate mixing in the circulation with minimum extravascular diffusion and metabolic loss.

TABLE I. SUMMARY OF PATIENTS STUDIED

GROUP AND PATIENT	SERUM			URINE	
	TOTAL PROTEIN (GM.%)	ALBUMIN (GM.%)	CHOLESTEROL (MG.%)	PROTEIN	DRB*
Group A. Patients with latent nephrosis (little or no edema and minimal hypoalbuminemia)					
R. T. (BIH 30111), 30-year-old white man, studied in October, 1955. Diagnosis: chronic glomerulonephritis	5.1	3.3	407	3+	+
J. C. (BIH 70382), 35-year-old white man, studied in October, 1955. Diagnosis: chronic glomerulonephritis	5.4	3.4	400	4+	+
H. G. (BIH 65760), 30-year-old white man, studied in June, 1957. Diagnosis: diabetes mellitus	(October, 1956) 4.6	2.0	445	4+	+
	(June, 1957) 6.3	3.4	330	4+	+
Group B. Patients with overt nephrosis (edema and moderate hypoalbuminemia)					
F. S. (BIH 65073), 29-year-old white man, studied in March, 1956. Diagnosis: subacute glomerulonephritis	4.3	1.4	340-400	4+	–
H. L. (BIH M 87292), 66-year-old white woman, studied in June, 1957. Diagnosis: nephrotic syndrome of undetermined etiology	4.6	1.3	1,100-1,630	4+	+
M. K. (BIH M 85228), 50-year-old white woman, studied in June, 1957. Diagnosis: nephrotic syndrome of undetermined etiology	4.3	2.1	520	4+	–

*Doubly refractile bodies.

All counting was done with samples in test tubes placed in a well-type sodium iodide crystal scintillation counter giving approximately 10^6 counts per minute per microcurie of I^{131}.

Albumin in the urine and serum at the beginning, middle, and end of the study was determined immunochemically using rabbit antiserum[4] and spectrophotometric analysis of the precipitates.[5]

RESULTS

Plasma Disappearance of I^{131}-Labeled Albumin.—The plasma disappearance curves are shown in Fig. 1. The interpretation of such curves has received

considerable attention.[6, 7] The initial rapid decline over the first few days represents exchange of the injected I^{131} albumin with available albumin in the body as well as loss via the kidneys and catabolism. The subsequent slower decline over the remaining days to weeks represents disappearance due to the latter two factors. The constant rate of the slower decline on a semilogarithmic plot indicates that a constant fraction of the tracer remaining in the body each day is lost as a result of two processes—catabolism and excretion in the urine. Assuming the tracer behaves identically to endogenous albumin, then a similar constant fraction of the endogenous exchangeable pool is catabolized and lost via the kidneys each day.

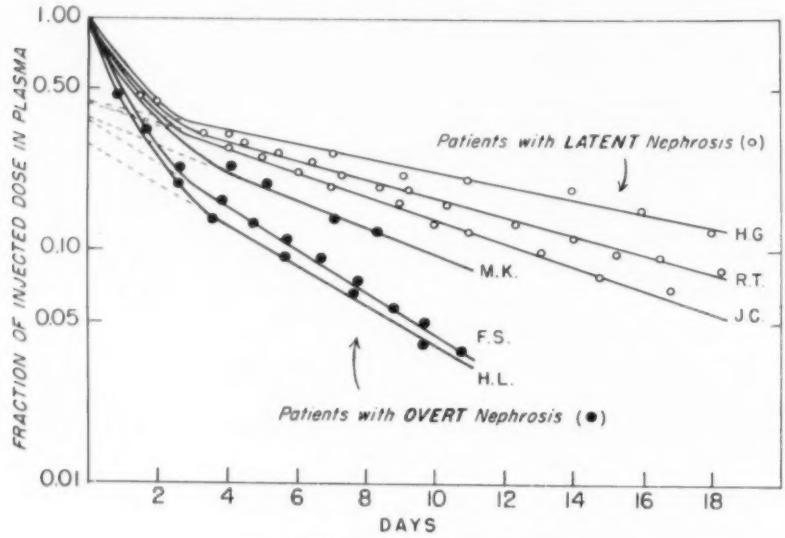

Fig. 1.—Plasma disappearance curves.

The results of graphic analysis of the plasma disappearance curves to determine the rates of disappearance of the tracer for these patients are shown in Table II. The normal range of turnover half-life for I^{131}-labeled albumin in adults is 10 to 17 days, corresponding to a turnover rate of 4 to 7 per cent of the body pool per day.[6-8] Since in the normal individual urinary loss is negligible, these figures represent rates of catabolism.

For Patients R. T. and J. C., who were edema-free, and H. G., with a trace of edema, these curves approach normal. The zero time intercept represents the fraction of the administered tracer that would have been present in the plasma had the tracer assumed its final volume of distribution initially. For these 3 patients, the intercepts are 0.42, 0.43, and 0.44; that is, the plasma I^{131} albumin would have been 42 to 44 per cent of the total I^{131} albumin if equilibrium had been obtained initially. This is in excellent agreement with the value

obtained by others of 0.45 as the fraction of the total exchangeable albumin pool that is in the plasma.[6-8]

TABLE II. I^{131} ALBUMIN TURNOVER DATA FROM PLASMA DISAPPEARANCE CURVES

PATIENT	ZERO-TIME INTERCEPT	HALF-LIFE (CATABOLISM PLUS URINARY LOSS) (DAYS)	RATE OF CATABOLISM PLUS URINARY LOSS* (PER CENT PER DAY)
Group A. Latent Nephrosis			
1. R. T.	0.43	7.3	9.5
2. J. C.	0.44	6.0	11.5
3. H. G.	0.42	10.5	6.6
Group B. Overt Nephrosis			
1. F. S.	0.38	3.0	23.0
2. H. L.	0.30	3.3	21.0
3. M. K.	0.38	5.0	13.9
Normal[6-8]	0.45	10 to 17 days	4 to 7% per day (Catabolism)

*Rate (per cent per day) $= \frac{0.693}{\text{half-life}} \times 100$.

In contrast to these patients, the more rapid disappearance of the I^{131}-labeled albumin from the plasma in the more severely nephrotic patients (F. S., H. L., and M. K.) is evident in Fig. 1. Since the disappearance curve represents the combined loss from albuminuria and catabolism it is not possible to determine their separate contributions from the plasma curves alone. The zero time intercepts of 0.30 and 0.38 in these nephrotic patients are much lower than the normal estimate of 0.45 as the fraction of the total albumin in the plasma, suggesting in nephrosis a higher than normal extravascular-intravascular ratio of albumin. An excessive amount of extravascular albumin in nephrosis is unlikely since nephrotic edema fluid contains so little albumin.[3] The overestimation of extravascular albumin in nephrosis has been ascribed to the fact that the specific activity of the I^{131} albumin becomes higher in the edema fluid than in the plasma. This is presumably due to the slow return of the tracer to the circulation from edematous portions of the body. In Patient M. K. serial sampling of the edema fluid from the legs revealed that the specific activity of the edema fluid was twice the plasma specific activity after 5 days.

In edematous patients, therefore, the zero time intercept represents the final volume of distribution of the tracer and not of the endogenous albumin. Under these circumstances the plasma disappearance curves cannot be reliably used to calculate fractional rates of catabolism and urinary loss or to estimate the relative amounts of endogenous albumin in the vascular and extravascular compartments.

On the other hand, the urinary data provide a more reliable method of calculation for the rates of albuminuria, catabolism, and synthesis of albumin since the fraction of the amount of albumin synthesized per day which is lost in the urine is the same as the fraction of the administered tracer dose of I^{131}-labeled albumin cumulatively recovered as I^{131}-labeled albumin in the urine.[3] With patients in a steady state in relation to albumin metabolism the rate of albumin synthesis should equal the rate of albuminuria plus the rate of catabolism.

The rates of albuminuria, catabolism, and synthesis of albumin as calculated from the equations[3] are shown in Table III. A_u, the quantity of albumin in the urine per day, was determined immunologically at the beginning, middle, and end of the study, and these values were averaged. F_p', the fraction of the injected I^{131} activity appearing in the urine as I^{131} albumin, was obtained by subtracting the measured, cumulative nonprotein I^{131} activity from the total urinary I^{131} activity recovered as fractions of the administered dose. It is necessary to calculate *cumulative* recoveries since the daily clearance of nonprotein I^{131} iodide may be delayed with rapid degradation of I^{131}-labeled albumin, especially in the presence of renal disease.

TABLE III. RATES OF URINARY LOSS, CATABOLISM, AND SYNTHESIS OF ALBUMIN*

PATIENT	URINARY LOSS (A_U) (GM. PER DAY)	F_p'	SYNTHESIS (A_S) (GM. PER DAY)	CATABOLISM (A_C) (GM. PER DAY)
Group A. Latent Nephrosis				
1. R. T.	4.1	0.27	15.2	11.1
2. J. C.	7.1	0.40	17.8	10.7
3. H. G.	4.55	0.23	19.7	15.3
Group B. Overt Nephrosis				
1. F. S.	13.5	0.48	28.1	14.6
2. H. L.	5.8	0.66	8.9	3.1
3. M. K.	6.6	0.55	12.1	5.5

*$A_S = \frac{A_U}{F_p'}$ and $A_C = A_S - A_U$.

The absolute amounts of albumin synthesized, catabolized, or lost in the urine daily as shown in Table III are variable and not well correlated with the clinical state of the patient. In order to compare these various rates of albumin metabolism among the patients one must take into account the marked differences in the total amount of albumin in the body. To determine the *fractional* rates of catabolism and urinary loss, that is, the fractions of the albumin in the body catabolized and lost in the urine daily, requires an estimate of the size of the total body pool of albumin. The total pool of albumin has been estimated using the expression:

$$\text{total albumin pool} = \frac{\text{plasma pool}}{0.45},$$

the relationship in nonedematous individuals. The plasma pool is the plasma volume obtained by isotope dilution multiplied by the plasma albumin concentration. The fractional rate of catabolism or urinary loss is the absolute rate of catabolism or urinary loss divided by the estimated total albumin pool. These data are shown in Table IV.

From Table IV it is apparent that in only one patient, F. S., is there a marked increase in the fractional rate of catabolism of albumin. In the other patients, both with latent and overt nephrosis, the fractional rates of catabolism are within normal limits.

In the other edematous nephrotic patients, H. L., and M. K., the increased fractional rate of urinary loss appears to account for their severe albumin

deficiency. However, from Table IV it would appear that the absolute rates of synthesis of albumin, 8.9 and 12.1 Gm. per day for Patients H. L. and M. K., respectively, are far below the value of 28.1 Gm. per day for Patient F. S. with a comparable degree of albumin deficiency. In Patients H. L. and M. K., therefore, impaired albumin synthesis may be a contributing factor to their albumin deficiency.

TABLE IV. ESTIMATED TOTAL ALBUMIN POOL AND FRACTIONAL RATES OF CATABOLISM AND URINARY LOSS OF ALBUMIN

PATIENT	PLASMA VOLUME (LITERS)	PLASMA ALBUMIN (GM./100 ML.)	PLASMA POOL (GM.)	TOTAL POOL (GM.)	FRACTIONAL RATE OF CATABOLISM (FRACTION PER DAY)	FRACTIONAL RATE OF URINARY LOSS (FRACTION PER DAY)
Group A. Latent Nephrosis						
1. R. T.	2.32	3.63	85.1	189	0.059	0.022
2. J. C.	3.01	3.32	100	222	0.048	0.032
3. H. G.	2.95	3.40	100	222	0.069	0.020
Group B. Overt Nephrosis						
1. F. S.	2.84	1.45	41.2	91.5	0.160	0.148
2. H. L.	2.98	1.09	32.5	72.4	0.043	0.081
3. M. K.	2.21	2.00	44.2	98.1	0.056	0.067
Normal	3.0	4.0	120	267	0.04 to 0.07	0.0004

These data indicate that the adult patients with *latent* nephrosis do not have a metabolic abnormality of albumin metabolism in that the fractional rate of catabolism is normal and the amount of albumin synthesized daily is sufficient to compensate for the urinary loss with little, if any, reduction in the total pool of albumin. In contrast, one of the patients with recent *overt* nephrosis and mild edema during the study has a markedly increased fractional rate of catabolism contributing as much to the increased albumin turnover and albumin deficiency as the massive urinary loss. In the other two patients with overt nephrosis, the fractional rate of catabolism is normal and urinary loss is considerable; albumin synthesis in these patients appears insufficient to overcome the urinary loss, resulting in a marked reduction in the total body pool of albumin.

DISCUSSION

Since this study involves assumptions concerning the validity of I^{131}-labeled albumin as a tracer for endogenous albumin, some data bearing on this problem will be reviewed. Turnover studies in human beings have been reported[9-12] using albumin labeled with I^{131}, C^{14}, and S^{35}, and the rates of degradation of albumin have not always agreed by these methods. The S^{35} and C^{14} labels introduce the problem of reincorporation of the degraded activity whereas I^{131} degraded from albumin appears entirely as nonprotein iodide in the urine when thyroid uptake is blocked. A crucial factor in determining the turnover of I^{131}-labeled albumin is the degree of iodination. Excessive iodination, above a few atoms of iodine per molecule of albumin, leads to a more rapid degradation.[7] Denaturation resulting in an increased rate of degradation may occur from self-radiation.[13]

Despite these problems, if the same or similar preparations of I^{131}-labeled albumin as were used were catabolized somewhat faster than endogenous albumin, there still would be a fixed relation between I^{131}-labeled and endogenous albumin metabolism. The differences found among the patients in this study would remain real, although the quantitative estimates of the various rates of metabolism might be in error.

The patients were assumed to be in a steady state as regards albumin metabolism so that the rate of albumin synthesis would remain equal to the rate of catabolism plus urinary loss throughout the study period. The criteria upon which this assumption is based were the negligible (less than 10 per cent) changes in serum albumin concentrations, urinary albumin, and body weight and an unchanged clinical status during the study.

Trapping of I^{131}-labeled albumin may lead to a higher specific activity of albumin in edema fluid compared to plasma. The extrapolated value for the ratio of plasma albumin to total exchangeable albumin from the plasma die-away curve would be too low under these circumstances. For the calculation of fractional rates in the patients with overt nephrosis the value of 0.45 as the ratio of plasma albumin to total albumin found in normal individuals was used instead of the observed lower values of 0.30 to 0.38. Even if the lowest value of 0.30 were used for Patient F. S., his total albumin pool would be increased from 91.5 to 137 Gm. and the fractional rate of catabolism would drop from 0.16 to 0.11. This would still represent a markedly accelerated fractional rate of catabolism compared to the normal range of 0.04 to 0.07.

The problem of unequal specific activities in edematous patients receiving I^{131}-labeled albumin has been reviewed by Kushner and associates[14] who found a threefold higher specific activity of edema fluid albumin compared to plasma in a patient with nutritional anasarca. Equal specific activities were found in 2 other edematous patients, 1 with nephrosis. In the one patient whose edema fluid was sampled in this study (Patient M. K.), the specific activity of the edema fluid albumin was twice that of the plasma after 5 days. One of the factors that may account for the discrepancy in specific activities among investigators is the method used for the determination of albumin when present in small quantities. Hypoproteinemic edema fluid of the type seen in nephrosis may have albumin concentrations as low as 10 mg. per cent. This amount can be accurately determined by the immunologic method using rabbit antiserum but concentration techniques and electrophoresis may lead to an overestimation of the albumin content and hence a lower specific activity.

In contrast to the findings in a study of children with the nephrotic syndrome,[3] in whom excessive catabolism was uniformly found with severe albumin depletion and anasarca, only 1 of the 3 adult nephrotic patients with fairly severe hypoalbuminemia had an excessive fractional rate of catabolism. However, these adult patients showed neither as much edema nor as severe albumin deficiency as the children classified as severe cases with marked anasarca and serum albumin levels of 0.5 Gm. per 100 ml. or less, in whom catabolism and urinary loss of albumin were both excessive. The adults with overt nephrosis

in this study are more comparable clinically to the children (in Group II) with *moderate* albumin depletion and some edema, in whom excessive catabolism was not uniformly observed. It is interesting that the adult patient in this study with the increased fractional rate of catabolism of albumin was much younger than the other 2 overtly nephrotic patients with normal fractional rates of catabolism. In the study of children with nephrosis, albumin synthesis was always in the high normal range; in contrast, among these adults impaired synthesis of albumin may have been present in the 2 oldest with overt nephrosis. In these patients, H. L. and M. K. (66 and 50 years old, respectively), the usual liver function tests were normal. M. K. received Meticorten during the study; however, it is unlikely that steroid therapy significantly influenced the results, since the patient showed no clinical change and levels of albumin in the serum and urine were the same before, during, and after steroid therapy.

In an earlier study of I^{131}-labeled albumin in nephrotic adults, Blahd and co-workers[15] interpreted the results as indicating no abnormality of catabolism. Their methods and calculations were not comparable to the present study, and it is not clear how rates of albumin synthesis and catabolism could be determined from a metabolic nitrogen balance and plasma I^{131} albumin die-away plots alone.

The mechanism of excessive catabolism of albumin in the nephrotic syndrome, when present, is not known. Abnormal catabolism is independent of the total amount of albumin in the body, since the fraction of the albumin pool catabolized by Patients M. K. and H. L. was normal; yet, for Patient F. S. with a comparable total pool the fraction catabolized was far above normal. The kidney has been implicated as a possible site of the normal degradation of proteins.[16] The role of the kidney in the excessive catabolism of albumin found in some nephrotic patients has not yet been established.

SUMMARY

1. I^{131}-labeled albumin turnover studies were carried out in 3 adult patients with latent nephrosis and 3 with overt nephrosis. One of the 6 adults, a 30-year-old man with hypoalbuminemia and mild edema, showed a striking increase in the fractional rate of catabolism of albumin which contributed as much to the depletion of the body pool of albumin as did the massive urinary loss. In this patient the rate of albumin synthesis was increased.

2. Some impairment of albumin synthesis may have been a contributing factor to the albumin deficiency in the 2 other cases of overt nephrosis in whom the rate of catabolism of albumin was normal. These 2 patients were 50 and 66 years old, respectively.

3. Rates of albumin catabolism and synthesis were normal in the 3 patients with latent nephrosis.

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CHANGES IN BODY COMPOSITION DURING THE COURSE OF ACUTE ANTERIOR POLIOMYELITIS

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THE important role potassium plays in various physiologic processes has been documented in several recent reviews[1-6]; however, relatively few studies of potassium metabolism during the course of acute anterior poliomyelitis had been reported prior to the initiation of our investigation, and the conclusions drawn from the observed data were contradictory. Earle[7] found no correlation between serum potassium and severity of paralysis, and the administration of potassium chloride to his paralyzed patients did not appear to improve muscle function. In contrast, Lans and associates[8] reported that the administration of potassium chloride to 3 patients suffering from bulbar poliomyelitis with hypokalemia produced marked improvement in their clinical condition. Bower and his colleagues[9, 10] measured serum potassium concentration and the excretion of potassium in the urine during the course of acute anterior poliomyelitis. They found the excretion of potassium in the urine to be consistently increased, while the serum potassium was elevated if the patients were "dehydrated" and/or oliguric, and normal or decreased if the patients "were adequately hydrated." They concluded an intracellular deficit of potassium developed during the course of acute anterior poliomyelitis and this deficit was indicated by the presence of hypokalemia, provided the patient was adequately hydrated and was not oliguric. In view of these conflicting conclusions we elected to study serially the intracellular potassium content, as defined by isotopic dilution techniques,[11] of patients suffering from acute anterior poliomyelitis.

METHODS

Subjects.—The 45 subjects described in this report were selected from the group of patients hospitalized in the Municipal Contagious Disease Hospital (MCDH) during the 1956 poliomyelitis epidemic in Chicago. This patient population comprised approximately 75 per cent of the individuals who had a diagnosis of acute anterior poliomyelitis reported to the city health authorities during the epidemic. Tables I and II include pertinent clinical data. Age was recorded to the nearest birthday. Onset of illness was measured from the date of the initial symptom recorded in the chart by the examining physician at MCDH. Because evaluation of muscle strength is difficult during the acute illness, the Lovett method for evaluating paralysis was simplified for the purposes of this study in the following manner: 3 plus, at

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most, complete range of motion with gravity eliminated; 2 plus, complete range of motion against gravity with minimal resistance; plus, complete range of motion against gravity with moderate resistance; minus, complete range of motion against gravity with full resistance. Severity of illness was arbitrarily evaluated by giving one plus point for each of the following factors: a day of fever, paralysis in each extremity for each extremity, paralysis of the trunk, paralysis of cranial nerves, catheterization of the bladder, tracheotomy, nasopharyngeal suction, and each complication. Methods of treatment have been previously described.[12] It was not possible to evaluate fluid or electrolyte balance during the course of the disease; however, in acutely ill patients the daily water and electrolyte intake was limited to 500 ml. of 5 per cent dextrose in water, 100 ml. of isotonic sodium chloride, and 3.0 Gm. of potassium chloride. Sodium and potassium salts were not administered during the days of the study. Patients not in respirators were weighed nude on a cart scale to an accuracy of ±0.1 Kg. It was not possible to perform serial measurements on all of the patients admitted to the study because the patients who did not develop complications were discharged after the acute phase of their illness passed. The study was limited to adults and large adolescents for the following reasons: (1) older patients are more likely to develop severe illness and, therefore, would be more apt to show changes in intracellular potassium content, if any occur, and (2) the need for generous amounts of blood for analysis. All of the patients studied serially were critically ill and the majority of them required continuous care in a tank respirator. None of the subjects were pregnant.

Procedure.—Studies were initiated twice a week, usually on Monday and Wednesday. Patients were incorporated into the investigation shortly after their admission to the hospital. Serial studies, when performed, were done at 7- to 10-day intervals. Approximately 200 μc of K^{42} as potassium carbonate* was injected in a potassium and sodium chloride solution, in a quantitative manner previously described[13] between 9:30 and 10:30 A.M. of the first day. All urine subsequently voided was collected and the collection period closed just prior to the quantitative injection of radiosulfate† and antipyrine‡ between 7:30 and 8:30 A.M. of the following day. Urine was quantitatively collected 1½, 3, 4½, and 6 hours after the injection of radiosulfate and antipyrine for the determination of total exchangeable potassium and radiosulfate space. Blood samples were obtained 3, 4, 5, and 6 hours after injection of radiosulfate and antipyrine for the determination of radiosulfate and antipyrine spaces. The methods for determination of radiosulfate space and antipyrine space have been reported.[14] In an appreciable number of patients, extrapolation of the radiosulfate counts excreted in the urine to zero time gave a number larger than the actual counts injected. When this occurred actual counts injected were used to compute the radiosulfate space. Serum and urine samples were frozen and stored until analyses of antipyrine and assay of sulfate radioactivity were to be done.

Total exchangeable potassium was computed by the following formula:

$$K_e = \frac{\text{counts } K^{42} \text{ injected} - \text{counts } K^{42} \text{ excreted in urine}}{\text{specific activity of urine sample}}.$$

Radioactivity of K^{42} was assayed in duplicate in a well-type scintillation counter to a counting error less than 1 per cent. Radiosulfate activity was not detected by the scintillation counter. Quadruplicate assay of standards of K^{42} and of radiosulfate was done in all cases. Standards were prepared by diluting the same volume (5 ml.) injected into patients to produce a counting rate approximating that observed in the urine of the subjects. Potassium was measured by a flame photometer. The mean K_e was determined by averaging the K_e obtained from the 4 urine samples. The coefficient of variation for the 69 determinations of total exchangeable potassium was $\overline{C} = 1.86 \pm 1.53$ per cent. The coefficient

*Prepared by irradiation in the reactor at the Argonne National Laboratories, Lemont, Ill., on allocation from the AEC.

†Obtained from Abbott Laboratories, North Chicago, Ill., by license from the AEC.

‡Generously supplied by Dr. B. L. Martz, Eli Lilly & Company, Indianapolis, Ind.

of variation of the 242 duplicate chemical determinations of antipyrine was C = 0.473 ± 0.513 per cent. In 16 samples, sufficient serum was not available for duplicate analysis for antipyrine. The following symbols and abbreviations have been employed:

$(K)_s$	= Serum potassium concentration
K_e	= Total exchangeable potassium
TBW	= Total body water (antipyrine space)
ECF	= Extracellular fluid volume (radiosulfate space)
ICF	= Intracellular fluid volume = TBW - ECF
LBM	= Lean body mass = TBW/0.73
B. Wt.	= Body weight
K_{LBM}	= Potassium content of lean body mass = K_e/LBM
ECFK	= Extracellular content of potassium = ECF × $(K)_s$
ICFK	= Intracellular content of potassium = K_e - ECFK
K_{ICF}	= Intracellular water concentration of potassium = ICFK/ICF
K_e/B. Wt.	= Total exchangeable potassium per kilogram of body weight
μ	= Mean (bar over variable denotes mean)
σ	= Standard deviation using N-1
M	= Male
F	= Female
M_A	= Initial studies of all males
F_A	= Initial studies of all females
F_N and M_N	= Serial studies where N denotes the number of the study
C	= Coefficient of variation
r	= Coefficient of correlation
t	= time

RESULTS

The measured and derived data for males are presented in Table I and for females in Table II. A summary of the means ± 1 standard deviation of selected measured and derived variables is presented in Table III.

Clinical Data.—There were no significant differences ($P > 0.05$) between the male and female patients with respect to age, the time after onset of the disease the studies were done, and the severity of illness. The differences in distribution by race within each sex were not significant by the χ^2 test ($P > 0.05$).

Serum Potassium.—$[\overline{K}]_s$ was within normal limits for all groups. There were no significant differences ($P > 0.05$) between $[\overline{K}]_s$ of corresponding subgroups of males and females. There were no significant differences ($P > 0.05$) between $[\overline{K}]_s$ of each of the subgroups of males compared to each other or of each of the subgroups of females compared to each other. In addition there was no significant ($P > 0.05$) correlation between $[K]_s$ and K_{ICW} or K_{LBM}.

Total Exchangeable Potassium.—Fig. 1 shows the regression of K_e on LBM for all of the initial studies of the males and females, respectively. Statistical analysis of the slope of each line indicates there is a significant regression ($P < 0.001$) of K_e on LBM. Statistical analysis of the intercept of each line indicates that the intercept does not significantly ($P > 0.05$) differ from zero.

TABLE I. CLINICAL, EXPERIMENTAL, AND DERIVED DATA OF MALE SUBJECTS

PATIENT	AGE	RACE	SEVERITY OF ILLNESS*	B. WT. (KG.)	DAY AFTER ONSET†	SERUM (K) (mEq./L.)	K_e (mEq.)	TBW		ECF		ICF (L.)	K_{ICF} (mEq./L.)	LBM (KG.)	K_{LBM} (mEq./KG.)	ECF_{TBW}	ICF_{TBW}	ECF_{ICF}	K_e/B. WT. (mEq./KG.)
								L.	% B. WT.	L.	% B. WT.								
K. P.	13	W	4	56.4	14	3.65	2385	37.3	66.1	12.2	21.6	25.1	93.2	51.1	46.7	0.33	0.67	0.49	42.3
D. G.	19	N	36	75‡	17	4.15	2851	32.2	–	15.0	–	17.2	162	44.1	64.6	0.47	0.53	0.87	–
–				–	27	4.60	2310	34.0	–	14.2	–	19.8	113	46.6	49.6	0.42	0.58	0.72	–
–				–	33	4.30	2304	33.2	–	16.1	–	17.1	131	45.5	50.6	0.49	0.51	0.94	–
E. St.	21	W	44	98‡	7	4.42	4110	39.6	–	20.0	–	19.6	205	54.3	75.7	0.51	0.49	1.06	–
–				–	14	4.08	3266	45.8	–	33.9	–	11.9	263	62.7	52.1	0.74	0.26	2.90	–
–				–	25	4.90	2285	37.5	–	19.3	–	18.2	120	51.4	44.5	0.51	0.49	1.10	–
J. K.	14	W	3	65.8	7	5.25	2313	25.7	39.1	10.1	15.4	15.6	145	35.2	65.7	0.39	0.61	0.65	35.1
H. P.	27	N	27	55‡	10	3.40	2035	25.2	–	6.5	–	18.7	108	34.5	59.0	0.26	0.74	0.35	–
–				–	19	3.75	1859	28.0	–	6.9	–	27.1	87	38.4	48.4	0.25	0.75	0.33	–
G. E.	17	W	1	67.1	7	3.70	3237	38.2	57.0	12.5	18.7	25.7	124	52.3	61.9	0.33	0.67	0.49	48.2
H. S.	25	W	9	78.1	7	4.82	3580	39.4	50.5	13.5	17.3	25.9	136	54.0	66.3	0.34	0.66	0.52	45.8
C. L.	22	N	15	–	12	5.00	3161	30.8	–	11.0	–	19.8	157	42.2	74.9	0.36	0.64	0.56	–
W. G.	34	N	14	89.5	4	3.40	4644	42.0	46.9	14.7	16.4	27.3	168	57.5	80.8	0.35	0.65	0.54	51.9

T. M.	28	N	30	68*	5	3.95	2493	31.2	–	12.2	–	19.0	129	42.7	58.4	0.39	0.61	0.64	–
–				–	19	4.10	2208	31.8	–	11.7	–	20.1	108	43.6	50.6	0.37	0.63	0.58	–
J. P.	13	W	31	–	7	4.05	1647	21.4	–	6.2	–	15.2	107	29.3	56.2	0.29	0.71	0.41	–
–				–	16	3.00	1293	24.0	–	8.0	–	16.0	79	32.9	39.3	0.33	0.67	0.50	–
–				–	28	2.90	967	17.3	–	6.8	–	10.5	90	23.7	40.8	0.39	0.61	0.65	–
–				30	37	4.52	1126	19.2	64.0	8.7	29.0	10.5	104	26.3	42.8	0.45	0.55	0.83	37.5
J. L.	23	W	6	62.8	4	3.60	3169	27.5	43.8	10.6	16.8	16.9	185	37.7	84.1	0.39	0.61	0.63	50.5
J. Ro.	24	W	7	68.1	5	3.65	2569	34.0	50.0	9.5	13.9	24.5	103	46.6	55.1	0.28	0.72	0.39	37.7
A. G.	21	W	2	59.0	9	3.65	3005	31.9	54.0	12.2	20.7	19.7	150	43.7	68.8	0.38	0.62	0.62	50.9
J. E.	20	N	3	64.2	5	3.88	3883	34.1	53.1	11.2	17.5	22.9	168	46.7	83.2	0.33	0.67	0.49	60.5
H. B.	26	W	6	62.5	6	3.82	3025	31.1	49.7	11.4	18.2	19.7	151	42.6	71.0	0.37	0.63	0.58	48.4
Em. W.	21	N	17	54.4	6	3.65	2877	36.4	67.0	11.1	20.4	25.3	112	49.9	57.7	0.31	0.69	0.44	52.9
J. Ra.	29	N	15	–	8	3.86	3323	32.3	–	11.6	–	20.7	158	44.3	75.0	0.36	0.64	0.56	–
–				65.1	15	4.18	2566	35.0	53.8	12.1	18.7	22.9	110	47.9	53.6	0.35	0.65	0.53	39.4
M. M.	32	N	8	75.1	6	3.60	3051	35.7	47.5	13.2	17.6	22.5	134	48.9	62.4	0.37	0.63	0.59	40.6
L. D.	28	W	0	84.5	7	3.95	3447	40.5	47.9	14.4	17.0	26.1	130	55.5	62.1	0.36	0.64	0.55	40.8

*See text for method of computation.
†From time of onset to initial study.
‡Last known body weight.

TABLE II. CLINICAL, EXPERIMENTAL, AND DERIVED DATA OF FEMALE SUBJECTS

PATIENT	AGE	RACE	SEVERITY OF ILLNESS*	B. WT. (KG.)	DAY AFTER ONSET†	SERUM (K) (mEq./L.)	K_e (mEq.)	TBW		ECF		ICF (L.)	K_{ICF} (mEq./L.)	LBM (KG.)	K_{LBM} (mEq./KG.)	ECF_{TBW}	ICF_{TBW}	ECF_{ICF}	K_e/B. WT. (mEq./KG.)
								L.	% B. WT.	L.	% B. WT.								
M. P.	18	W	26	64‡	14	–	1901	–	–	–	–	–	–	–	–	–	–	–	–
D. B.	26	W	11	78.3	7	–	1528	–	–	–	–	–	–	–	–	–	–	–	19.5
–				77.2	24	4.25	1573	23.6	30.5	7.9	10.3	15.7	98.1	32.3	48.7	0.33	0.67	0.50	20.4
K. G.	9	W	14	40.1	17	4.42	1272	19.5	48.6	8.3	20.8	11.2	110	26.2	48.5	0.43	0.57	0.74	31.7
–				39.2	31	5.40	1311	17.8	45.5	8.0	20.3	9.8	129	24.4	53.7	0.45	0.55	0.82	33.4
C. F.	14	W	8	35.9	28	4.18	1362	19.3	53.9	6.8	18.9	12.5	107	26.4	51.6	0.35	0.65	0.54	37.9
M. He.	12	N	3	32.7	10	5.40	1315	19.1	58.5	6.6	20.2	12.5	102	26.2	50.2	0.35	0.65	0.53	40.2
R. Z.	33	W	30	45‡	13	3.63	969	16.7	–	6.4	–	10.3	93	22.9	42.3	0.38	0.62	0.62	–
–				–	23	4.10	1130	17.7	–	11.1	–	6.6	164	24.3	46.5	0.63	0.37	1.68	–
–				–	29	4.78	1067	15.6	–	8.5	–	7.1	145	21.4	49.9	0.55	0.45	1.20	–
M. Ha.	28	W	16	50.6	11	4.27	1810	22.1	43.6	12.5	24.7	9.6	183	30.3	59.7	0.57	0.43	0.30	35.8
L. C.	12	N	13	36	17	4.42	1651	20.9	58.0	6.3	17.6	14.6	111	28.6	57.7	0.30	0.70	0.43	45.9
H. C.	23	W	4	67.6	6	4.25	1829	23.9	35.3	8.6	12.7	15.3	117	32.7	55.9	0.36	0.64	0.56	27.1
J. B.	27	W	10	49.8	12	3.75	1974	25.6	51.4	15.8	31.8	9.8	195	35.1	56.2	0.62	0.38	1.61	39.6
E. So.	40	W	10	91.0	9	3.70	2399	32.8	36.0	10.8	11.9	22.0	107	44.9	53.4	0.33	0.67	0.49	26.4
M. F.	22	W	0	71.3	5	4.00	2149	29.3	41.1	9.6	13.4	19.7	107	40.1	53.6	0.33	0.67	0.49	30.1
B. G.	26	W	8	50.7	7	3.95	1859	25.8	50.9	9.1	18.0	16.7	109	35.3	52.7	0.35	0.65	0.55	36.7
B. V.	25	W	9	61.5	21	4.48	1615	24.3	39.5	8.2	13.3	16.1	98	33.3	48.5	0.34	0.66	0.51	26.3
E. H.	23	W	3	54.0	11	4.48	1933	25.0	46.2	9.0	16.7	16.0	118	34.3	56.4	0.36	0.64	0.56	35.8
M. B.	24	W	10	51.5	7	3.28	2085	25.8	50.2	7.8	15.1	18.0	114	35.3	59.1	0.30	0.70	0.43	40.5
M. J.	27	W	39	–	13	3.18	1330	25.4	–	10.1	–	15.3	85	34.8	38.2	0.40	0.60	0.66	–
–				–	20	3.40	1394	23.8	–	11.2	–	12.6	108	32.6	42.8	0.47	0.53	0.89	–
–				–	34	4.10	1174	20.1	–	8.8	–	11.3	101	27.5	42.7	0.44	0.56	0.78	–

L. S.	13	W	36	–	6	3.48	1667	24.1	–	9.4	–	14.7	111	33.0	50.5	0.39	0.61	0.64	–
–				–	13	4.25	1490	20.3	–	7.1	–	13.2	111	27.8	53.6	0.35	0.65	0.54	–
–				41.4	20	4.66	1143	18.3	44.2	9.4	22.7	8.9	124	25.1	45.5	0.51	0.49	1.06	27.6
B. A.	24	W	40	54‡	5	3.86	1797	23.7	–	10.2	–	13.5	130	32.5	55.3	0.43	0.57	0.76	–
–				–	12	3.65	1569	23.0	–	9.0	–	14.0	110	31.5	49.8	0.39	0.61	0.64	–
–				–	24	4.18	1332	19.4	–	8.4	–	11.0	118	26.6	50.1	0.43	0.57	0.76	–
El. W.	28	N	56	–	8	4.23	2027	26.4	–	9.4	–	17.0	117	36.2	56.0	0.36	0.64	0.55	–
–				–	15	3.82	1845	23.9	–	9.9	–	14.0	129	32.7	56.4	0.41	0.59	0.71	–
–				–	27	3.52	1478	24.7	–	13.0	–	11.7	122	33.8	43.7	0.53	0.47	1.11	–
G. M.	31	N	6	77.4	10	4.90	2547	26.5	34.2	9.8	12.7	16.7	150	36.3	70.2	0.37	0.63	0.59	32.9
A. T.	18	N	4	52.8	4	4.10	1947	24.5	46.4	7.5	14.1	17.0	113	33.6	57.9	0.31	0.69	0.44	36.9
R. S.	24	W	26	–	5	3.82	2380	27.6	–	7.4	–	20.2	116	37.8	63.0	0.27	0.73	0.37	–
–				–	12	4.70	1823	29.4	–	14.1	–	15.3	115	40.3	45.2	0.48	0.52	0.92	–
–				–	19	4.00	1806	27.6	–	10.7	–	16.9	104	37.8	47.8	0.39	0.61	0.63	–
P. J.	26	W	21	–	12	3.40	1165	20.6	–	10.5	–	10.1	112	28.2	41.3	0.51	0.49	1.04	–
B. P.	21	W	2	48.0	6	4.10	1855	27.4	57.1	10.7	22.4	16.7	108	37.5	49.5	0.39	0.61	0.64	38.6

*See text for method of computation.
†From time of onset to initial study.
‡Last known body weight.

TABLE III. SUMMARY OF $\mu \pm \sigma$ OF SELECTED EXPERIMENTAL AND DERIVED DATA

MALES	INITIAL STUDIES ALL SUBJECTS (20)*	SERIAL STUDIES 1 (6)*	2 (6)*	3 (3)*	4 (1)*	MIXED
$[K]_s$ (mEq./L.)	3.97 ± 0.52	3.97 ± 0.31	3.95 ± 0.54	4.03 ± 1.04	4.52	–
K_{LBM} (mEq./Kg.)	66.5 ± 10.2	64.8 ± 8.7	48.9 ± 5.1	45.3 ± 5.0	42.8	–
K_{ICW} (mEq./Kg.)	141 ± 29.3	145 ± 37.8	127 ± 68.2	114 ± 21.2	104	–
K_e/B. Wt. (mEq./Kg.)	46.6 ± 7.1†	–	–	–	37.5	38.5 ± 1.3‡
ECF_{TBW}	0.36 ± 0.059	0.38 ± 0.098	0.41 ± 0.17	0.46 ± 0.07	0.45	–
ICF_{TBW}	0.64 ± 0.059	0.62 ± 0.098	0.59 ± 0.17	0.54 ± 0.07	0.55	–
ECF_{ICF}	0.57 ± 0.16	0.64 ± 0.26	0.93 ± 0.97	0.90 ± 0.23	0.83	–
Day after onset study done	7.7 ± 3.4	9 ± 4.2	18 ± 4.7	29 ± 3.5	37	–
Severity of illness	13.9 ± 13	30.5 ± 9.6	–	–	–	–
FEMALES	ALL (23)*	1 (7)*	2 (8)*	3 (6)*	–	–
$[K]_s$ (mEq./L.)	4.06 ± 0.52	3.80 ± 0.41	4.20 ± 0.63	4.21 ± 0.46	–	–
K_{LBM} (mEq./Kg.)	53.4 ± 7.7	50.5 ± 8.5	49.6 ± 4.7	46.6 ± 3.1	–	–
K_{ICW} (mEq./Kg.)	118 ± 24.7	109 ± 15.2	120.5 ± 20.5	119 ± 15.9	–	–
K_e/B. Wt. (mEq./Kg.)	34.2 ± 6.7§	–	–	–	–	27.1 ± 6.5‖
ECF_{TBW}	0.38 ± 0.084	0.38 ± 0.055	0.44 ± 0.094	0.47 ± 0.064	–	–
ICF_{TBW}	0.62 ± 0.084	0.62 ± 0.055	0.56 ± 0.094	0.53 ± 0.064	–	–
ECF_{ICF}	0.65 ± 0.29	0.62 ± 0.13	0.84 ± 0.37	0.92 ± 0.23	–	–
Day after onset study done	10.6 ± 6.0	9.6 ± 4.8	18 ± 7.1	25.5 ± 5.7	–	–
Severity of illness	16.0 ± 15	34.4 ± 13.1	–	–	–	–

*Parentheses indicate number of patients in study.
†Thirteen patients.
‡Two patients.
§Seventeen patients.
‖Three patients.

$\overline{K}_e$ decreased during the course of the illness; however, the decrease was not significant ($P > 0.05$). The line of regression of K_e on LBM was derived for each serial study of males and females. Statistical analysis of each line indicated that there was a significant regression ($P < 0.05$) of K_e on LBM for each line and the intercept of each line did not differ significantly from zero ($P > 0.05$).

LBM Content of Potassium (Fig. 2).—The initial $\overline{K}_{LBM}$ for all males was 66.5 mEq./Kg. and for all females 53.4 mEq./Kg. The difference is significant ($P < 0.001$). The difference between males and females is significant for the initial serial studies ($P < 0.025$). The males showed a significant decrease in K_{LBM} during the course of the illness as indicated in Table IV. A significant decrease ($P < 0.05$) in K_{LBM} of females was present only between the initial study of all of the female patients and the third serial study of the female patients. When the subjects were classified by race the pattern was the same; however, the differences between males and females were significant only for the white subjects ($P < 0.001$).

TABLE IV. PROBABILITY VALUES OBTAINED BY COMPARING $\overline{K}_{LBM}$ OF MALES AND $\overline{K}_{LBM}$ OF EACH SUBGROUP OF MALES

SUBJECTS COMPARED	P
$M_A - M_1$	> 0.05
$M_A - M_2$	< 0.001
$M_A - M_3$	< 0.005
$M_1 - M_2$	< 0.005
$M_1 - M_3$	< 0.01
$M_2 - M_3$	> 0.05

Intracellular Water Potassium Concentration.—The initial $\overline{K}_{ICF}$ for all males was 145 mEq./L. and for all females 118 mEq./L. This difference is significant ($P < 0.01$). A significant difference ($P < 0.05$) was also demonstrated between the $\overline{K}_{ICF}$ of males and of females in the first serial study. Other differences are not significant ($P > 0.05$). During the course of the illness $\overline{K}_{ICF}$ for males decreased; however, the decrease was not significant statistically ($P > 0.05$). There was little change in $\overline{K}_{ICF}$ for females during the course of the illness.

Exchangeable Potassium Per Kilogram of Body Weight.—The initial $\overline{K}_e$/B. Wt. for males was 46.6 mEq./Kg. and for females 34.2 mEq./Kg. The difference is significant ($P < 0.001$). K_e/B. Wt. could be computed for a few patients studied serially and decreased during the course of the illness. The difference between males and females in the repeat studies is significant ($P < 0.05$).

Body Water.—Between the initial and second study TBW increased 9.1 per cent in males and decreased 4.4 per cent in females. This difference is significant ($P < 0.05$). By the third serial study TBW had decreased below the initial value in males and there was an additional decrease in the females. There is no significant difference in the distribution of water between the extracellular

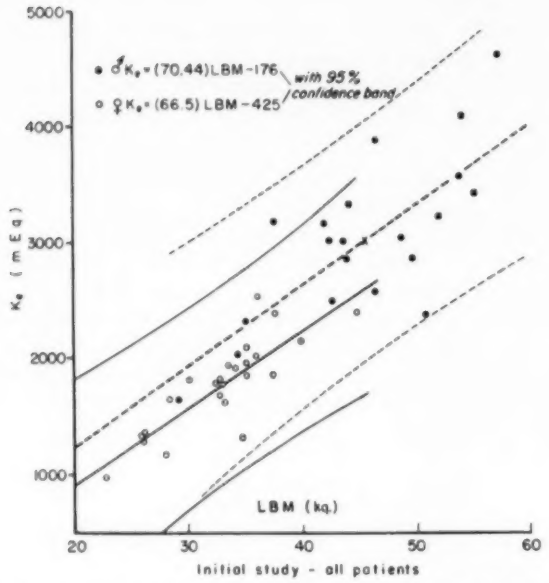

Fig. 1.—Regression of total exchangeable potassium upon lean body mass.

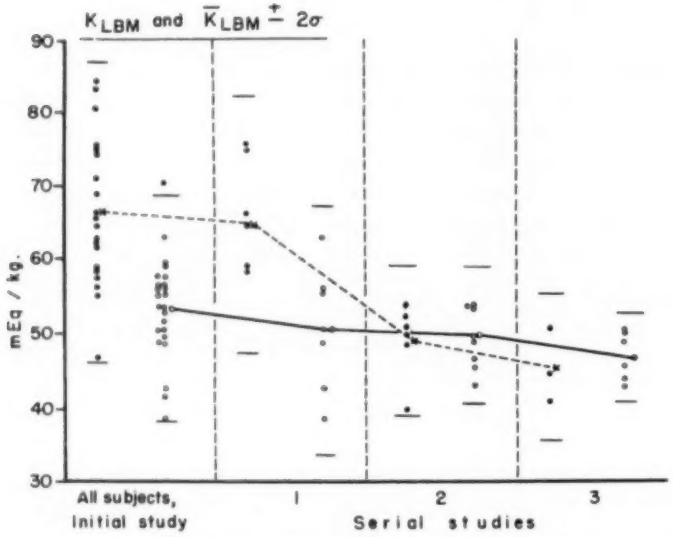

Fig. 2.—Lean body mass content of potassium.

and intracellular water compartments when the mean values for corresponding subgroups of males and females are compared ($P > 0.05$). During the course of the illness there is a change in the distribution of water, ECF is increased and ICF is decreased; this change is significant in males ($P < 0.005$) and females ($P < 0.05$).

Single Serial Study (Fig. 3).—One of the male patients (postpubertal) studied serially had considerable recovery of muscle function and was able to leave the respirator. It is of interest to note that his clinical course was associated with hypokalemia, decreased K_{ICW}, and a decreased K_{LBM}. All of these increased toward values found in clinically healthy subjects (see Discussion), with clinical improvement. The changes in body water distribution were characteristic of those observed for the group as a whole. K_e decreased during the illness and increased with recovery.

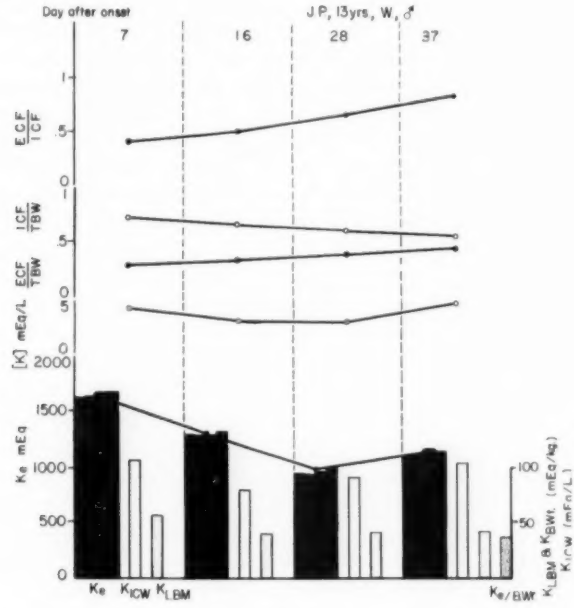

Fig. 3.—Changes in body composition obtained by serial study of Patient J. P.

DISCUSSION

We have elected to employ the lean body mass model of Behnke[15] as modified by Pace and his co-workers[16-18] for reasons that will be evident later in the discussion. Keys and Brozek[19] have two criticisms of the lean body mass model: (1) the inclusion of the indefinite concept of essential lipids into the model and (2) the belief that the composition of the lean body mass model is

not independent of the total fat content of the body. However, the concept of essential lipids does not affect our use of the LBM model and their belief that body fat significantly affects the composition of the lean body mass is based on computational errors. They give correlation coefficients on page 282 of reference 19, computed from the data of Pace and his colleagues[16-18] which, they state, show a significant correlation between total body fat and percentage of water in the fat-free body. Our own computations from these data give $r_M = 0.3082$ and $r_F = 0.3575$; these correlation coefficients are not significant. Since the intercept of each of the regression lines does not differ significantly from zero, we can replace the line defined by the equation $K_e = b$ (LBM) + constant, by the line $K_e = b$ (LBM), compute b for each group and compare the results. Evidence supporting this extrapolation of the regression lines will be cited later in the discussion. Granting the previous assumptions, it is evident that we have demonstrated a significant difference in potassium metabolism between males and females during the course of acute anterior poliomyelitis. In females there is little change in K_{ICW} and a significant (when F_3 is compared to F_A) decrease in K_{LBM} during the course of the illness. In males, K_{ICW} and K_{LBM} decrease during the course of the illness; however, the decrease is significant only for K_{LBM}. By the third serial study, K_{LBM} and K_{ICW} are not significantly different for males and females. The changes for K_{ICW} are presumably not as clear-cut as for K_{LBM} because of the larger experimental error introduced into the calculation of K_{ICW} in accordance with the law of the propagation of errors. That these differences are not secondary to changes in (1) extracellular content or concentration of potassium is suggested by the lack of any significant correlation of ECFK or $[K]_s$ with K_{ICW} or K_{LBM}, or (2) water metabolism is suggested by the occurrence in males and females of identical changes in the distribution of water despite the fact that TBW increased in males and decreased in females between the first and second serial studies, and the difference between the per cent change in TBW of males and females was significant. The validity of this interpretation of our data depends upon the validity of the proposition that there is no difference between males and females in the chemical composition of body tissue, at least with respect to potassium content. We have not been able to study clinically healthy subjects to evaluate this point. Other evidence to evaluate the proposition is meager but is available and is derived from the following sources: (1) analysis of whole adult cadavers, (2) analysis of tissue samples, (3) isotopic dilution studies, and (4) measurement of K^{40} content of human beings.

Analysis of Whole Adult Cadavers.—The reported data and their source are presented in Table V.[20-22] If we discard the 48-year-old man who was wasted and edematous and if, in addition, we assume that the fat-free body model and the lean body mass model are identical, we find nothing here to contradict our hypothesis. If the higher K_{LBM} of the female analyzed does not represent a sampling error and the K_{LBM} of females is greater than the K_{LBM} of males, then our hypothesis is further reinforced by these data, since the changes we have found are in the opposite direction.

TABLE V. BODY CONTENT OF POTASSIUM OBTAINED BY DIRECT ANALYSIS OF CADAVERS

REFERENCE	SEX	AGE	K (mEq./KG.) FAT-FREE BASIS
Shohl: Mineral Metabolism, 1939, Reinhold Publishing Corporation	Adult (Patient 19, Table III)	–	66.8
	Adult (Patient 20, Table V)	–	68
Widdowson and associates: *Clin. Sc.* 10: 113, 1951	M	25	71.1
	F	42	72.6
	M	48	33.5
Forbes and Lewis:	M	46	66.5
J. Clin. Invest. 6: 596, 1956	M	60	66.6

Analysis of Body Tissue.—A recent comprehensive review[23] of the water and electrolyte content of various body tissues does not consider possible sex differences in the chemical composition of tissues. A compilation of biologic data[24] does not subclassify the data, pertaining to chemical composition of body tissues, by sex. Investigators with extensive experience in the analysis of muscle[25] and nervous tissue[26] have never demonstrated any sex differences in the electrolyte content of these tissues. Other workers fail to designate the sex of control subjects in their published work[27-33] and combine males and females in the statistical analysis of data. When the sex is given, a significant difference in the potassium content of liver[21] and muscle[34-37] cannot be demonstrated. The situation is not quite so clear-cut for blood. Sex differences in serum electrolyte concentration have not been demonstrated.[38] While some workers have demonstrated a significantly higher red cell content of potassium in women,[39] other investigators using better sampling techniques have not been able to confirm that a difference exists.[40] In summary, most workers have assumed sex differences do not exist or have not been able to demonstrate any sex difference in the potassium content of liver, brain, skeletal muscle, and blood.

Isotopic Dilution Studies.—Table VI summarizes isotopic dilution data published by other workers[41-52] for clinically healthy and hospital control adults.

K_c/B. Wt.: The wide spread of the data reported for adults for each sex may be due to one or all of the following factors: (1) sampling errors, (2) differences in experimental technique, or (3) true differences in body composition or potassium metabolism of individuals from different geographical regions. Most authors attribute the difference between sexes to a difference in the content of body fat.

K_{LBM}: The lean body mass model and the fat-free body model can be considered to be identical for the purposes of our discussion for the incorporation of the concept of essential lipids into the LBM model[15] does not change the sense of our analysis. Except for the data of Ikkos,[47, 50] the data of different investigators are in good agreement for K_{LBM} of men. The significantly lower K_{LBM} for women in these studies is surprising in view of the

TABLE VI. BODY CONTENT OF POTASSIUM FROM ISOTOPIC DILUTION STUDIES

REFERENCE	MALES					FEMALES				
	NO. OF SUBJECTS	AGE IN YEARS	$\overline{K}_e$/B. WT. (mEq./KG.)	$\overline{K}_{LBM}$ (mEq./KG.)	$\overline{K}_{ICW}$ (mEq./L.)	NO. OF SUBJECTS	AGE IN YEARS	$\overline{K}_e$/B. WT. (mEq./KG.)	$\overline{K}_{LBM}$ (mEq./KG.)	$\overline{K}_{ICW}$ (mEq./L.)
Corsa and associates: *J. Clin. Invest.* 29: 1280, 1950	29 12	25.8 ± 3.3 25.4 ± 3.5	46.3 ± 4.3 –	– 55.9 ± 3.5	– 125 ± 11	– –	– –	– –	– –	– –
Deane and Smith: *J. Clin. Invest.* 31: 197, 1952	5	31.4 ± 12.3	41.5 ± 3.2	56.0 ± 3.5	111.6 ± 11.1	1	18	32.9	49.8	113
Aikawa and associates: *J. Clin. Invest.* 31: 367, 1952	–	–	–	–	–	20	24.1 ± 3.2	31.5 ± 2.9	–	–
Arons and co-workers: *J. Clin. Invest.* 33: 1001, 1954	5	28.0 ± 1.4	51.1 ± 2.7	–	–	7	23.6 ± 2.4	42.5 ± 3.7	–	–
Blainey and associates: *Clin. Sc.* 13: 165, 1954	17	44.9 ± 12.0	45.9 ± 15.0	–	–	7	40.7 ± 11.1	35.0 ± 13.0		
Moore and co-workers: *Metabolism* 3: 334, 1954	–	–	–	–	–	14	23.6 (19-29)*	39.0 ± 5.4		
Ikkos and co-workers: *Metabolism* 4: 231, 1955	9	30.2 ± 7.1	50.6 ± 9.3	67.5 ± 4.3	127.1 ± 6.9†	5	28.4 ± 7.7	38.7 ± 4.3	58.2 ± 6.7	113.7 ± 14.4†

Rundo and Sagild: *Nature* 175: 774, 1955	6	28.2 ± 4.2	48.6 ± 2.2	–	–	4	21.2 ± 5.0	42.7 ± 2.9		
Corsa and associates: *Pediatrics* 17: 184, 1956	1	0.083 (premature)	40.0	–	–	–	–	–	–	–
	8	0.82 ± 0.82	37.5 ± 3.7	38.6 ± 3.0‡	–	–	–	–	–	–
	2	11.0 ± 5.9	40.0 ± 1.4	45.3 ± 1.3	–	5	10.2 ± 3.0	38.5 ± 9.4	46.6 ± 3.6§	–
Ikkos and co-workers:‖ *Acta Endocrinol.* 21: 211, 1956	13	30.4 ± 8.5	50.2 ± 6.2	61.6 ± 3.3	116.9 ± 6.9**	19	25.8 ± 6.9	37.9 ± 4.2	55.4 ± 4.2	107.6 ± 8.7**
James and Robertson: *A. M. A. Am. J. Dis. Child.* 93: 217, 1957 (Sex not stated)	7	5.9 ± 2.2	44.1 ± 2.9	57.2 ± 6.5	–	–	–	–	–	–
Muldowney and associates: *J. Clin. Invest.* 36: 1375, 1957	13	29.4 ± 10.1	45.7 ± 6.2	57.2 ± 4.7	–	10	48.2 ± 17.8	30.1 ± 3.6	46.3 ± 7.2	–

*Individual ages not listed.
†Using TBW_{AP} and ECW_{IN} for Subjects 1 to 12; TBW_{AP} and ECW_{TS} for Subjects 13 and 14.
‡Seven subjects.
§Four subjects.
‖Probably includes subjects reported in 1955.
**Using ICW_{In}.

fact that in our studies K_{LBM} was experimentally identical in males and females by the third serial study. In the study of Muldowney and his co-workers[52] this difference could be due to the difference in age of the male and female subjects, for age variations in muscle electrolyte composition have been described.[53] Corsa and co-workers[41] have stated K^{42} exchanges with 95 per cent of the body potassium. A limited number of measurements of K^{42} and K^{40} in the same subject by Rundo and Sagild[48] has indicated $\frac{K^{42}}{K^{40}} = 85$ per cent. If we apply this ratio to the available data for K^{42}_{LBM} for males we find, with the exception of the results of Ikkos,[47] the corrected K^{42}_{LBM} data to be in fairly good agreement with the results obtained from the direct analysis of cadavers. For females the data of Ikkos are of the right order of magnitude while Muldowney's data are low. As mentioned previously we cannot explain these discrepancies at the present time.

Experimental support for the extrapolation of the regression line toward the origin is available in Table VI. The data for infants indicate a K_{LBM} somewhat lower than that found for adults. However, this is probably due to the appreciably larger extracellular fluid volume found in infants.[54] Correction of the K_{LBM} data for infants for this enlarged ECF brings their values into the adult range.

K_{ICW}: The variation in K_{ICW} for each sex can be accounted for in part by the use of substances, for the measurement of body fluid compartments, with noncongruent volumes of distribution. However, the sex difference in Ikkos' data is significant ($P < 0.05$) and is as surprising as his K_{LBM} data, for there was no significant ($P > 0.05$) difference in K_{ICW} between our male and female subjects by the third serial study.

K^{40} Content of Humans.—A number of workers[48, 55-61] have been interested in the K^{40} content of humans. Burch and Spiers[60] have summarized much of the data: for males, 30 mEq./Kg. $< K^{40}$/B. Wt. $<$ 55 mEq./Kg.; for females, 30 mEq./Kg. $< K^{40}$/B. Wt. $<$ 51 mEq./Kg. On the basis of fat-free weight the potassium content of the body appears to be independent of gross weight, age, and sex and is estimated to be 71.6 mEq./Kg. of fat-free weight.[58, 61] This value is probably the most reliable estimate of the fat-free body content of potassium, for the studies have been limited to clinically healthy individuals. In addition, it would indicate a ratio for $\frac{K^{42}}{K^{39}}$ in the neighborhood of the 85 per cent indicated by Rundo and Sagild,[48] and somewhat lower than the 95 per cent suggested by Corsa and associates.[41]

In conclusion, we would interpret the available evidence as indicating an identical body content and tissue concentration of potassium in clinically healthy males and females. One may question the validity of an estimation of the composition of tissue of clinically healthy subjects obtained by extrapolation from the data of our third serial study. However, in an experimental model similar in many respects to our clinical situation, Eichelberger[62] has shown that

in muscle the intracellular concentration of potassium does not change and the extracellular fluid volume increases after denervation. Unfortunately she did not study the dynamics of the change.

Our observation of an increase in K_{LBM} and K_{ICW} in males can be used to explain the interesting results of Howard[63, 64] and Whedon and Shorr[65] of potassium losses considerably less than the theoretical losses expected during balance studies. Our failure to observe any significant correlation between $[K]_s$ and K^{42}, K_{LBM}, or K_{ICW} confirms the observations of others[66]; however, we did not measure serum pH or pCO_2 as have others.[67, 68] Our failure to do so may have precluded the observation of any relationship between $[K]_s$ and pH, pCO_2, K_e^{42}, K_{LBM}, or K_{ICW}. Other studies of ours indicate a relationship between LBM and $[K]_s$ and because of the linear relationship between K_e and LBM suggest a relationship between K_{LBM} and $[K]_s$.[69]

The water metabolism of the patients studied serially is of interest. Rigid fluid and sodium restriction was enforced for these patients despite the fact that they were acutely ill with high body temperatures, had continuous nasopharyngeal suction, and were encased in tank respirators—conditions which presumably contributed to the induction of maximal fluid losses. Clinical studies[12] of patients during previous epidemics suggest that the presence of pulmonary edema at post mortem was associated with the conventional regimen of 3 L. of water and 8.5 Gm. of NaCl administered daily. The demonstration of an actual increase in the TBW in the males and the small decrease in TBW in the females between the first and second studies despite the rigid fluid restriction provides experimental support for the previous clinical conclusions. Unfortunately, the physical conditions of the epidemic precluded clear-cut documentation of this point in terms of fluid balance. However, the large quantities of water that can be produced by catabolism of body tissues has been documented in other conditions.[14, 70] The changes in water distribution are identical to those that have been described in undernutrition.[71, 72] This suggests that these changes in water distribution may be one facet of a general biologic response to stress. The use of the LBM model assumes that the ratio $\frac{\text{cell mass}}{\text{total body water}}$ remains constant. It is evident that this was not so in our studies. The application of any correction factor to take this into account would accent the differences in K_{LBM} and K_{ICW}, in view of the fact that initially the TBW for males increased while the TBW for females decreased.

There remains for discussion the biologic interpretation of our observations. Analysis of the epidemiologic and clinical data indicates that young men are less susceptible to infection with poliomyelitis virus, have a higher incidence of complications and have a higher mortality than nonpregnant young women.[73-78] The simplest assumption to account for these differences is to relate them to the different steroid hormones present in the internal environment of males and females. Support for this proposition as a first approximation is obtained from the work of Teodoru and Shwartzman, who have been able to modify the susceptibility to infection with poliomyelitis virus of the Golden Syrian hamster

(normally a resistant animal) by suitable alteration of testicular and adrenocortical function.[79-82] Certain of their experiments suggest that simultaneous administration of testosterone and cortisone to hamsters increases the paralytic incidence and mortality following intracerebral inoculation of MEF virus, when compared to control, cortisone-treated, testosterone-treated, and castrated animals.[79, 83] The important role that changes in the physicochemical environment of the tissues of the host play in the modification of the host-parasite relationship has been recently reviewed and discussed by Dubos.[84] The importance of hormonal factors in this scheme is well illustrated by the enhanced susceptibility of women to infection with poliomyelitis virus produced by pregnancy.[74, 76]

Studies are in progress to delineate further the changes in the internal environment that occur during the course of acute central nervous system infections.

SUMMARY

1. Total exchangeable potassium, antipyrine space, and radiosulfate space were simultaneously determined in 44 patients suffering from acute anterior poliomyelitis. In a selected number of patients the measurements were repeated at 7- to 10-day intervals.

2. A significant difference in potassium metabolism between males and females was demonstrated. In males the intracellular potassium concentration was increased early in the illness, while in females it remained unchanged during the course of the illness.

3. A significant difference in the metabolism of water between males and females was demonstrated. In males total body water increased early in the illness while in females it decreased during the course of the illness. The distribution of water between intracellular and extracellular compartments was identical in males and females. During the course of the illness the extracellular fluid compartment expanded and the intracellular fluid compartment contracted.

4. The biologic interpretation of these sex differences in potassium and water metabolism is discussed.

We would like to express our thanks to Dr. Mark Lepper for calling to our attention the need for a study of potassium metabolism during the course of acute anterior poliomyelitis. We are particularly grateful to the nursing staff of the Municipal Contagious Disease Hospital for their unstinting cooperation during a trying period. The senior author would like to thank Mr. Paul Gunther, statistical consultant to the Wright Air Development Command and to Caywood-Schiller Associates, for critical comments about the statistical notions involved in the analysis of the data. We would like to thank Drs. Josef Brozek, Mark Lepper, Robert Kark, S. Howard Armstrong, Jr., and Harry Dowling for reviewing the manuscript.

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THE EFFECT OF DIGITALIS AND DESOXYCORTICOSTERONE ACETATE ADMINISTRATION UPON INTRACELLULAR POTASSIUM ION CONCENTRATION

A Chemical and Morphologic Study

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THE theory of Szent-Gyorgi[1] on muscle contraction has drawn attention to the importance of potassium in intracellular physiology. Since the publication of his work, it has become increasingly evident that the actions of digitalis are intimately associated with the intracellular concentration of the potassium ion.[2]

It has been well documented that toxic doses of digitalis are capable of producing myocardial lesions in experimental animals.[3-5] Numerous investigators have indicated also that potassium deficiency results in similar morphologic changes both in animals and in man.[6-8] As a corollary to these studies it has been shown that potassium depletion in animals increases cardiac sensitivity to digitalis.[9-11] Robinson and associates[12] have recently stated that in potassium-deficient rats toxic doses of digitalis did not accentuate the development of myocardial lesions. These authors felt that digitalis neither added to nor protected from the effect of potassium deficiency in their animals.

In this study large doses of digitoxin alone and in combination with desoxycorticosterone acetate (DOCA) were administered to dogs. The results of a chemical and morphologic study of the myocardium of these animals form the basis for the present report.

METHODS

Mongrel dogs were used throughout the study. A standard laboratory diet was given, and water was allowed as desired. All drugs were administered by intramuscular injection daily except on Saturday and Sunday. The animals receiving digitoxin lost weight, became anorexic, and appeared toxic at the end of the experimental period.

The animals were divided into 4 groups.

Group I. Controls: Four dogs considered to be in good health.

Group II. DOCA Alone: Four dogs, 2 of which received 15 mg. of DOCA daily for a total dose of 150 mg. in 13 days and 2 dogs received a total dose of 225 mg. in 20 days.

Group III. Digitoxin Alone: Two dogs received 0.4 mg. daily for a total dose of 6 mg. in 20 days and 3 animals received 0.6 mg. daily for a total dose of 9 mg. in 20 days.

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Group IV. DOCA Plus Digitoxin: Five animals received 0.6 mg. digitoxin daily plus 15 mg. of DOCA daily for a total dose of 9 mg. of digitoxin and 225 mg. of DOCA in 18 to 21 days, respectively.

All animals were killed by ether anesthesia. Blood was removed immediately from the left ventricle by means of syringe and needle for estimations of serum sodium potassium and chloride. The hearts were then removed for the following pathologic and chemical studies.

Tissue blocks were taken from the anterior, lateral, and posterior ventricular walls, right ventricle, and interventricular septum. A minimum of 6 tissue blocks were selected from each heart. Tissues were fixed in 10 per cent formalin. Microscopic sections were cut at 6 micra and stained with hematoxylin and eosin and by the Hotchkiss-McManus periodic acid-Schiff stain.

Chemical analysis was performed on portions of the left ventricular myocardium in each case. Tissue specimens varied in weight between 0.5 to 1.0 Gm. One tissue specimen was dried in an oven at 110° C. to constant weight to determine dry weight. The dried tissue was then extracted with ether to determine fat content, fat-free wet muscle weight and fat-free dry muscle weight. In each case 5 additional muscle specimens were taken for chemical analysis. The values for each dog represent the mean of these 5 analyses. After weighing, each muscle specimen was homogenized in 50 ml. of distilled water using a Waring blender with microcell. After homogenization, the mixture was centrifuged at 3,000 r.p.m. for 10 minutes to produce a slightly opalescent flesh-colored fluid. Complete disintegration of muscle fibers was determined by microscopic examination of the homogenate. Chlorides were determined on a 20 ml. aliquot using the method of Whitehorn.[13] Sodium was determined on undiluted samples and potassium on a 1:25 dilution of the sample by means of flame photometry. A Beckman Model B spectrophotometer with flame attachments was used exclusively.

Extracellular water was determined from tissue chlorides and serum chloride according to standard methods and formulas.[14] The following symbols are used in the tables:

H_2O_m = grams of muscle water per kilogram of wet fat-free muscle.
Cl_m = mEq. of chloride per kilogram of wet fat-free muscle.
H_2O_e = extracellular phase in gram of water per kilogram of wet fat-free muscle.
H_2O_i = intracellular phase in grams of water per kilogram of wet fat-free muscle.
Na_i = mEq. of Na per kilogram of wet fat-free muscle.
K_i = mEq. of K per kilogram of wet fat-free muscle.
Na_{cw} = mEq. of Na per kilogram of intracellular water.
K_{cw} = mEq. of K per kilogram of intracellular water.
Na_s = serum Na in mEq. per liter of serum.
K_s = serum K in mEq. per liter of serum.
Cl_s = serum Cl in mEq. per liter of serum.

RESULTS

A. *Tissue Analysis Data.*—The noteworthy findings of Table I are the following:

(1) In Groups II (DOCA alone), III (digitoxin alone), and IV (DOCA plus digitoxin), H_2O_e is increased and H_2O_i is decreased. In these same groups K_i is decreased and K_s is increased. (2) In Groups III (digitoxin alone) and IV (DOCA plus digitoxin), K_{cw} is increased despite the decrease in K_i.

These data indicate that both DOCA and digitoxin produced a decrease in intracellular water and an increase in extracellular water in the myocardium. It is interesting to note that during the course of this study the serum potassium concentration rose. It should be emphasized that due to the decrease of

intracellular water the actual ionic concentration of intracellular potassium, K_{cw}, increased in the animals receiving digitoxin alone as well as in those receiving digitoxin plus DOCA.

TABLE I. SUMMARY OF THE MEAN RESULTS OF THE CHEMICAL STUDIES FOR ALL FOUR GROUPS

	GROUP I CONTROL		GROUP II DOCA ALONE		GROUP III DIGITOXIN ALONE		GROUP IV DOCA PLUS DIGITOXIN	
NO. DOG	4	VARIATION	4	VARIATION	5	VARIATION	5	VARIATION
H_2O_m	749	740-755	724	705-735	743	722-774	747	730-761
Cl_m	19.5	18.2-20.3	21.7	19.7-23.8	25.9	24.6-27.3	32.3	25.5-37.3
H_2O_c	152	146-157	168	153-179	199	184-209	240	188-278
H_2O_i	597	583-609	556	552-558	554	527-570	507	463-548
Na_i	7.6	4.7-11.1	10.3	9.0-12.5	8.1	6.4-12.3	9.0	4.9-10.7
K_i	81.3	76.1-83.6	75.3	72.5-78.1	78.0	72.1-82.5	71.7	61.5-79.5
Na_e	148	146-151	154	150-158	156	148-164	158	151-164
K_e	5.6	4.8-6.0	7.6	5.8-10.0	7.4	5.9-8.9	7.2	5.8-8.7
Cl_e	112	109-113	113	110-116	114	112-117	117	112-119
Na_{cw}	12.6	8.1-18.1	18.5	16.0-23.0	14.9	10.0-23.0	17.7	9.8-20.3
K_{cw}	136.6	125-143	136.0	130-143	143.5	134-153	141.0	125-169

B. *Morphologic Data.*—Microscopic examination of tissue from all animals revealed no characteristic changes. Several sections from each animal showed patchy areas of lymphocytic infiltrations, interstitial fibrosis and some minimal areas of cellular necrosis. These findings appeared occasionally in the control animals as in the other groups. The administration of DOCA and excessive doses of digitoxin and the chemical aberrations produced by these substances did not cause any significant degree of abnormal morphologic change.

DISCUSSION

From the results of this study it is apparent that DOCA alone and the combination of DOCA and toxic doses of digitoxin are capable of lowering intracellular potassium content. Digitoxin alone did cause a fall in intracellular potassium, but not to a marked degree. In contrast to our findings, Gardner[15] and his associates found in the rat that the combination of DOCA and strophanthin G did not lower intracellular potassium to as low a level as when either agent was used alone. These authors concluded that a metabolic antagonism exists between digitalis and potassium. This may be explained by the observation of Robinson[12] that the rat is resistant to the effects of digitalis. Zeeman and associates,[10] on the other hand, found that potassium-depleted dogs exhibited a marked sensitivity to lanatoside C, but were unable to demonstrate a definite decrease in cardiac muscle potassium content.

Most important in the present study is the fact that although intracellular potassium levels diminished, intracellular water content also diminished. This resulted in an actual increase in potassium ion concentration per unit of intracellular water in the dogs that received digitoxin alone and those that received digitoxin plus DOCA. Lown[2] has stated, "There is general agreement that toxic doses of digitalis liberate potassium from both skeletal and heart muscle." In our study, excessive doses of digitoxin did produce some decrease in total cardiac muscle potassium, but of greater significance was the actual increase in intracellular potassium ion concentration.

If it is ever permissible to correlate experimental data with clinical experience, the results of this study would imply that the effectiveness of potassium administration to patients suffering from digitalis intoxication may not be dependent upon the increase in the intracellular concentration of the potassium ion.

The lack of clear-cut morphologic changes in the myocardium following DOCA and digitoxin administration may be explained by the rather short duration of the experiment, and by the absence of protein depletion or the lack of an excessive sodium intake. These factors may be necessary concomitants for the production of potassium deficiency lesions in the dog.

SUMMARY

A study is presented in which groups of dogs were given injections of large doses of DOCA and digitoxin. Chemical analysis of the heart muscle of these animals revealed that DOCA alone, digitoxin alone, and a combination of these agents produced a drop in total muscle potassium. However, due to the decrease in intracellular water content, actual intracellular potassium ion concentration was increased in the animals receiving digitoxin alone and those receiving digitoxin plus DOCA.

The animal hearts were studied for morphologic lesions similar to those reported elsewhere, but no difference was found in the drug-treated animals from a group of normal controls.

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THE ABSORPTION OF VITAMIN B_{12} LABELED WITH RADIOACTIVE COBALT^{60} FOLLOWING SUBTOTAL GASTRECTOMY

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MEGALOBLASTIC anemia is an uncommon complication of a subtotal gastrectomy,[1, 2] although there have been reports of such an occurrence.[3-9] Recent reports indicate that impaired absorption of vitamin B_{12} occurs following subtotal gastrectomy, but is uncommon.[5, 9-12] We have performed a similar study of the absorption of Co^{60}-labeled vitamin B_{12} in a large group of patients who had had subtotal gastrectomies. In patients with impaired absorption of vitamin B_{12}, the effect of gastric stimulation with a secretagogue and the effect of the administration of an intrinsic factor preparation have been studied. In addition, we have investigated the role of the type of anastomosis involved in the production of the absorptive defect.

METHODS AND MATERIALS

The absorption of Co^{60}-labeled vitamin B_{12} was measured by means of a modification of the Schilling test,[13] the results being expressed as the per cent of the radioactivity of the oral dose (1 μg vitamin B_{12} labeled with 0.7 to 1.0 μc Co^{60}) excreted in the urine in 24 hours. In 16 normal subjects and in 17 patients with pernicious anemia the range of values for the 24-hour urinary excretion of radioactivity was found to be 6.5 to 22.3 per cent and 0.0 to 1.3 per cent, respectively. Ten patients with pernicious anemia had the Schilling test repeated with administration of intrinsic factor concentrate at the time of the oral dose of Co^{60}-labeled vitamin B_{12} and the range of values was found to be 4.0 to 19.7 per cent.

The absorption of vitamin B_{12} was measured in 34 patients who had undergone conventional subtotal gastrectomy from 1 to 70 months earlier. Fifteen patients had subtotal gastric resections with gastroduodenostomies, and 19 patients had subtotal gastric resections with gastrojejunostomies. Vagotomies had been performed in 2 patients in the former group and 4 patients in the latter. In both groups of patients, 60 to 80 per cent of the distal portion of the stomach had been resected, leaving a portion of the fundus in every case. Surgery was performed for peptic ulcer in 30 instances and for carcinoma in 4. Anemia was not present in any of the patients.

Four patients with subtotal gastrectomies were found to have an abnormally low urinary excretion of Co^{60}-labeled vitamin B_{12} comparable to values found in cases of pernicious anemia. Additional studies were performed on these patients as follows:

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The vitamin $B_{12}Co^{60}$ used in these studies was supplied through the courtesy of Dr. Augustus Gibson of Merck Sharp & Dohme, Rahway, N. J.

The intrinsic factor concentrate used in these studies was supplied through the courtesy of Dr. Kenneth W. Thompson of Organon, Inc., Orange, N. J.

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(1) a Schilling test with concomitant administration of 50 mg. of intrinsic factor concentrate; (2) a Schilling test 90 minutes after gastric stimulation with 50 mg. of Histalog (3-beta-aminoethyl pyrazole) administered subcutaneously[14]; (3) blood urea nitrogen and blood creatinine determinations; (4) plasma prothrombin and serum carotene levels. The latter procedures were performed to eliminate renal disease and small bowel malabsorption syndromes.

RESULTS

The results are presented in Tables I and II. The range of urinary excretion of Co^{60}-labeled vitamin B_{12} was 7.5 to 36.4 per cent in the group of 15 patients with subtotal gastric resection with gastroduodenostomy. Three patients had associated vagotomies.

The range of urinary excretion of Co^{60}-labeled vitamin B_{12} was 0.7 to 31.3 per cent in the group of 19 patients with subtotal gastric resection and gastrojejunostomy. Four of the patients in this group excreted less than 4.7 per cent of the administered radioactivity. In all 4 patients the urinary excretion of radioactivity increased to within the normal range when the Schilling test was repeated with 50 mg. of intrinsic factor concentrate. In 2 of these 4 patients urinary excretion of vitamin $B_{12}Co^{60}$ increased after Histalog administration, one rising to 6.4 per cent. Blood urea nitrogen, blood creatinine, plasma prothrombin concentration, and serum carotene determinations were normal in these patients.

DISCUSSION

Impaired absorption of vitamin B_{12}, as measured by the Schilling test of urinary excretion, was found in 11 per cent of a group of 34 patients who had had subtotal gastric resections. The patients who had impaired absorption of vitamin B_{12} were not anemic probably because they were studied too soon after gastrectomy to have depleted their body stores of vitamin B_{12}. Megaloblastic anemia is uncommon following subtotal gastric resection. MacLean in screening over 1,500 patients with subtotal gastrectomies found only 9 patients with megaloblastic anemia.[9]

The 4 patients with impaired absorption of vitamin B_{12} had had subtotal gastric resections with gastrojejunostomies. None of the 15 patients, who had subtotal gastric resections with gastroduodenostomies had impaired absorption of vitamin B_{12}. Twenty-five cases of megaloblastic anemia following subtotal gastric resection or gastrojejunostomy have been reported in the medical literature with details of the type of surgical anastomosis.[3-9] In all but one of these cases, the subtotal gastric resection has been associated with a gastrojejunostomy. In the one case associated with gastroduodenostomy, a high Billroth I resection was accomplished.[5] The 3 patients reported by Lowenstein[12] whose absorption of vitamin B_{12} was impaired after subtotal gastric resection also had had gastrojejunostomies.

These observations indicate that impaired absorption of vitamin B_{12} after subtotal gastric resection occurs predominately in patients who have had the stomach remnant anastomosed to the jejunum. Since the defect in absorption

TABLE I

PATIENT	AGE	DIAGNOSIS	MONTHS AFTER OPERATION	TYPE OF PROCEDURE	GASTRIC ANALYSIS BEFORE OPERATION	PER CENT URINARY EXCRETION OF VITAMIN $B_{12}Co^{60}$
						VITAMIN $B_{12}Co^{60}$ ALONE
J. J.	54	Gastric ulcer	19	Gastroduodenostomy	Free acid with histamine	11.0
E. N.	77	Duodenal and gastric ulcer	43	Gastroduodenostomy	Free acid	9.6
F. M.	50	Duodenal ulcer	39	Gastroduodenostomy and vagotomy	Free acid	21.5
C. W.	57	Carcinoma of stomach	1¼	Gastroduodenostomy	—	8.5
E. S.	65	Duodenal ulcer	21	Gastroduodenostomy	Free acid postoperatively	30.1
A. P.	60	Duodenal ulcer	30	Gastroduodenostomy	—	15.8
M. W.	50	Duodenal ulcer	6	Gastroduodenostomy	Free acid after histamine	11.7
B. H.	39	Duodenal ulcer	7	Gastroduodenostomy	—	10.4
M. H.	58	Carcinoma of stomach	19	Gastroduodenostomy	—	9.6
D. A.	56	Gastric ulcer	13	Gastroduodenostomy	—	13.0
W. B.	62	Gastric ulcer	2	Gastroduodenostomy	Free acid with histamine	7.5
P. D.	51	Gastric ulcer	20	Gastroduodenostomy	Free acid	20.0
W. M.	18	Gastritis, no ulcer	3	Gastroduodenostomy and vagotomy	Free acid	18.8
G. M.	35	Duodenal ulcer	16	Gastroduodenostomy	Free acid	24.6
J. R.	34	Duodenal ulcer	1¼	Gastroduodenostomy	—	36.4
C. P.	39	Pyloric ulcer	1	Gastrojejunostomy	Free acid	25.1
N. W.	42	Chronic gastritis	7	Gastrojejunostomy	—	9.7
B. A.	65	Duodenal ulcer	4	Gastrojejunostomy and vagotomy	Free acid	22.9
F. G.	82	Duodenal ulcer	2¾	Gastrojejunostomy	—	12.4
M. S.	56	Gastric and duodenal ulcer	6¼	Gastrojejunostomy	—	18.0
C. W.	48	Duodenal ulcer	4	Gastrojejunostomy	No free acid after histamine	11.7
C. C.	70	Gastric and duodenal ulcer	3½	Gastrojejunostomy	—	25.6
C. T.	65	Gastric ulcer	15¾	Gastrojejunostomy	Free acid after histamine	17.8
J. M.	58	Duodenal ulcer	28¼	Gastrojejunostomy	—	15.8
A. D.	47	Duodenal ulcer	5¾	Gastrojejunostomy	—	17.1
F. F.	61	Duodenal ulcer	4¾	Gastrojejunostomy	—	23.2
S. F.	49	Carcinoma of stomach	5	Gastrojejunostomy	—	31.3
J. B.	40	Duodenal ulcer	60	Gastrojejunostomy	—	10.5
E. G.	30	Duodenal ulcer	70	Gastrojejunostomy	—	8.2
G. D.	71	Duodenal ulcer	9	Gastrojejunostomy	—	18.5

of vitamin B_{12} can be corrected by the addition of intrinsic factor, the abnormality can be attributed to an inadequate quantity of intrinsic factor. Several factors undoubtedly contribute to this inadequacy. First, decreased gastric secretion of intrinsic factor may occur, because of an inadequate gastric phase of secretion. Stimulation of gastric secretion through the gastric mechanism can be invoked by mechanical distention of the pyloric antrum by food or by contact with the antral mucosa of chemical substances derived chiefly from meat. The resection of a large segment of the antrum associated with increased rate of gastric motility and emptying may decrease the effectiveness of this hormonal mechanism. In 2 of our cases with poor absorption of vitamin B_{12} stimulation of gastric secretion with a secretagogue resulted in improved absorption of vitamin B_{12}.

TABLE II

PATIENT	AGE	DIAGNOSIS	TIME IN MONTHS AFTER OPERATION	TYPE OF PROCEDURE	GASTRIC ANALYSIS BEFORE OPERATION	PER CENT URINARY EXCRETION OF VITAMIN B_{12} Co^{60} AFTER		
						VITAMIN B_{12} Co^{60}	PLUS INTRINSIC FACTOR	FOLLOWING 50 MG. HISTALOG, I.M.
C. R.	33	Duodenal ulcer; diverticulum of third portion of duodenum	1½	Gastrojejunostomy and vagotomy	Free acid	3.8	7.2	6.4
M. H.	60	Carcinoma of stomach	7	Gastrojejunostomy	—	0.7	7.9	0.7
T. S.	64	Duodenal ulcer	½ mo. with intrinsic factor; 1 mo. with Histalog	Gastrojejunostomy and vagotomy	—	4.7	10.4	4.6
L. R.	44	Duodenal ulcer	1¼	Gastrojejunostomy	—	1.8	12.4	5.6

In the 9 cases of megaloblastic anemia associated with subtotal gastric resection reported by MacLean,[9] he noted in the surgical specimens intestinalization of the gastric mucosa and considered this a cause for decreased secretion of intrinsic factor. Three of the 4 patients with decreased absorption of vitamin B_{12} who were studied in our laboratory had normal gastric mucosa in the surgical specimens. The fourth patient (M. H.) was found to have a gastric carcinoma with intestinalization of the gastric mucosa.

Second, following gastric resection with gastrojejunostomy the duodenum is bypassed, and the stomach contents enter directly into the jejunum. Although vitamin B_{12} in the presence of intrinsic factor is absorbed throughout the small intestine, the duodenum is undoubtedly one of the more important sites of vitamin B_{12} absorption. Citrin[15] and his associates, in studies on the site of absorption of vitamin B_{12} from the intestinal tract in normal subjects, obtained the highest urinary excretion values when vitamin B_{12} was instilled by tube

directly into the duodenum although significant absorption of vitamin B_{12} did occur following instillation into the jejunum and ileum. Observations of von Bonsdorff[16] in cases of megaloblastic anemia associated with fish tapeworm infestation also attest to the importance of the duodenum in vitamin B_{12} absorption. Dislodgment of the worms from the duodenum induced remission of the megaloblastic anemia even though the tapeworms remained in the lower portion of the intestinal tract.

Naish[17] and his associates reported a case of megaloblastic anemia following gastrojejunostomy and attributed the cause to intestinal stagnation in the afferent loop of jejunum. In our cases the blind loop syndrome is an unlikely explanation for the defect in absorption of vitamin B_{12} inasmuch as the defect was corrected by the simultaneous administration of intrinsic factor and vitamin B_{12}. In the presence of intestinal blind loops which interfere with vitamin B_{12} absorption, intrinsic factor does not correct the absorptive defect.

Intestinal malabsorption of vitamin B_{12} associated with steatorrhea also is unlikely in our patients inasmuch as intrinsic factor corrected the absorptive defect. In addition, the 4 patients had no diarrhea and maintained their weights. Plasma prothrombin and serum carotene values were normal in all cases.

Renal disease as a cause of an abnormally low urinary excretion of vitamin B_{12} was excluded since there was no history of renal disease, the urinalyses, blood urea nitrogen, and creatinine values were normal and the low urinary excretion of Co^{60}-labeled vitamin B_{12} returned to normal levels with the addition of intrinsic factor to the Schilling test.

SUMMARY

Vitamin B_{12} absorption was studied by means of a modified Schilling technique in 34 patients who had had subtotal gastric resection. Decreased absorption was found in 4 of the 19 patients who had had subtotal gastric resections with gastrojejunostomies. The deficiency was corrected by the administration of intrinsic factor. All of the patients with gastroduodenostomies absorbed vitamin B_{12} normally. Decreased gastric secretion of intrinsic factor and bypassing of the duodenum are considered important factors in the decreased absorption of vitamin B_{12} in the patients with subtotal gastrectomy associated with a gastrojejunostomy.

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D-XYLOSE AND POTASSIUM IODIDE ABSORPTION AND SERUM CAROTENE IN PERNICIOUS ANEMIA

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THE presence of an absorptive defect in pernicious anemia was initially demonstrated by Castle[1] who, in 1929, described the absence of intrinsic factor in the gastric juice. Citrin and associates,[2] instilling vitamin B_{12} directly into the small intestine, have shown this to be the normal site of B_{12} absorption. Employing a modified Schilling test, they further confirmed the inability of patients with pernicious anemia to absorb vitamin B_{12} in the absence of added intrinsic factor.

The question of impaired small intestinal absorption of other nutrients in pernicious anemia is raised by certain similarities of this disease to nontropical sprue. Megaloblastic anemia, glossitis, peripheral neuropathy, and low serum vitamin B_{12} levels are frequently encountered in sprue patients.[3-5] In addition, Gardner[6] has described similar alterations in gastric cytology in these two conditions.

Depression of serum carotene levels[7] and xylose absorption[8, 9] are found in the sprue syndrome. Heath and Fullerton[10] noted a delay in salivary iodide excretion following oral ingestion of potassium iodide in 7 of 14 patients with pernicious anemia, and concluded that this was evidence of impaired iodide absorption from the small intestine. It is the purpose of this report to evaluate further the absorptive activity of the small intestine in pernicious anemia.

MATERIALS AND METHODS

Serum carotene levels, blood xylose levels, and urinary excretion of xylose were measured in the following subjects:

1. Fifteen normal subjects between the ages of 25 and 72 years (Table I).
2. Twenty patients with pernicious anemia in hematologic remission, aged 55 to 85 years. The potassium iodide test was performed on 12 of this group (Table II).
3. Five pernicious anemia patients in hematologic relapse, the diagnosis in each case being confirmed by the characteristic bone marrow, low serum vitamin B_{12} levels, and a reticulocyte response to parenteral vitamin B_{12} therapy. Repeat studies were done following therapeutic remission (Table III). The potassium iodide test was also performed on 3 of these patients.

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4. Six patients with chronic nonmegaloblastic anemia, with hemoglobin levels comparable to those found in Group III (Table IV).

D-Xylose and Carotene Tests.—Following an overnight fast, the bladder was emptied and the urine discarded. Twenty-five grams of D-xylose, dissolved in 500 ml. of water, was given by mouth at 7 A.M. The xylose content of a 5-hour urine collection was determined. Patients remained fasting during the test. Blood was drawn for xylose and carotene determinations 2 hours following ingestion of the sugar. Xylose was determined by the method of Roe and Rice,[11] carotene by the method of Bessey and associates.[12]

Potassium Iodide Test.—The subjects drank a solution of 0.25 Gm. of potassium iodide in 50 ml. of water after an overnight fast. The mouth was then rinsed and saliva collected every 2 minutes in a cup containing 1 ml. of saturated starch solution and 4 drops of 10 per cent ferric chloride solution. The time elapsing between ingestion of potassium iodide and its appearance in saliva was measured. The presence of as little as 0.027 mg. of free iodine released by ferric chloride resulted in the appearance of a blue-black precipitate.

RESULTS

The five hour urinary xylose excretion of 10 normal subjects ranged from 3.7 to 7.9 Gm., with an average value of 5.71 Gm. (Table I and Fig. 1). The 2-hour blood levels varied from 18.2 to 42.2 mg. per cent, with an average of 31.8 mg. per cent (Table I and Fig. 2). These latter values are in accord with those reported by previous authors.[9, 13, 14] Serum carotene in the control group ranged from 55 to 192 μg per cent with an average of 124.8 μg per cent (Table I and Fig. 3), which is in agreement with normal values (50 to 420 μg per cent) reported by others.[7] Salivary iodide excretion time, after oral potassium iodide ingestion, in 45 healthy subjects varied from 11 to 16 minutes.

TABLE I. SERUM CAROTENE AND D-XYLOSE DETERMINATIONS IN CONTROL SUBJECTS

CASE	AGE	2-HOUR BLOOD XYLOSE (MG. PER CENT)	5-HOUR URINE XYLOSE (GM.)	SERUM CAROTENE (μG PER CENT)
1	25	34.4	6.7	192
2	28	–	–	167
3	28	–	–	178
4	29	42.2	3.7	–
5	30	36.4	7.6	108
6	30	22.6	7.9	–
7	34	–	–	123
8	36	21.5	6.8	–
9	40	18.2	3.8	–
10	40	39.7	3.8	–
11	45	–	–	55
12	50	33.8	5.6	–
13	58	–	–	65
14	60	37.8	7.5	116
15	72	32.2	3.7	119

Normal 2-hour blood xylose levels were obtained in all 20 pernicious anemia patients in remission (Fig. 2). Urinary xylose excretion was normal in 13 of this group; low values were noted in the remaining 7 (Cases 1 to 7, Table II). Serum carotene and salivary iodide excretion times were normal in all patients tested (Table II).

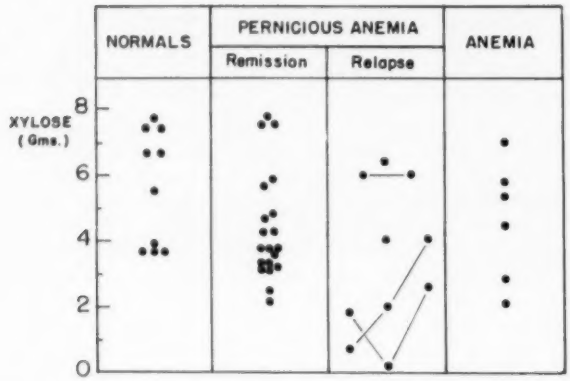

Fig. 1.—Five-hour urine xylose determinations in normal subjects and patients with pernicious and other anemias.

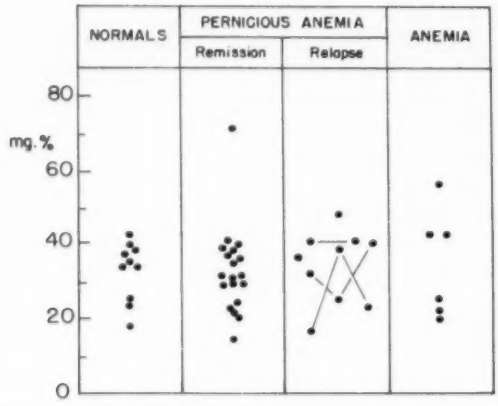

Fig. 2.—Two-hour blood xylose determinations in normal subjects and patients with pernicious and other anemias.

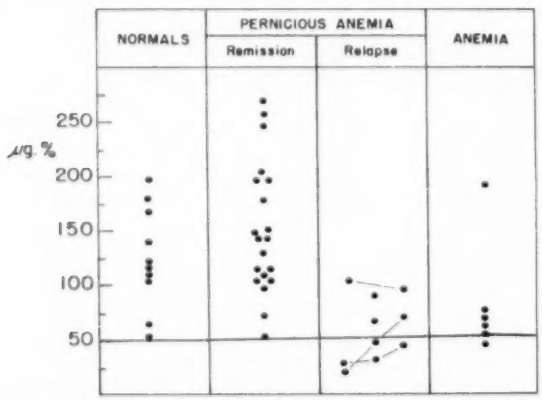

Fig. 3.—Serum carotene determinations in normal subjects and patients with pernicious and other anemias.

TABLE II. SERUM CAROTENE AND D-XYLOSE DETERMINATIONS IN PERNICIOUS ANEMIA PATIENTS IN REMISSION

CASE	AGE	HEMOGLOBIN	POTASSIUM IODIDE SALIVARY EXCRETION TIME (MINUTES)	2-HOUR BLOOD XYLOSE (MG. PER CENT)	5-HOUR URINE XYLOSE (GM.)	SERUM CAROTENE (μG PER CENT)
1. D. C.	72	14.0	12	*	2.1	115
2. H. F.	69	13.4	10	37.5	2.4	55
3. H. W.	77	12.5	15	23.8	3.4	99
4. C. S.	53	15.6	18	44.9	3.4	71
5. M. O'T.	54	14.1	12	40.7	3.3	265
6. G. M.	60	13.9	11	29.0	3.3	193
7. E. S.	71	12.5	10	29.9	3.2	139
8. G. S.	72	13.1	14	31.1	4.9	150
9. M. D.	55	13.2	12	35.5	5.9	115
10. R. B.	50	13.3	16	72.2	7.4	206
11. C. B.	65	14.3	14	40.0	4.7	131
12. J. C.	60	15.3	10	19.2	8.4	199
13. E. D.	71	14.2	–	21.5	5.8	138
14. J. G.	65	16.8	–	38.1	4.2	249
15. I. H.	50	14.5	–	32.1	3.8	148
16. M. L.	65	15.6	–	20.6	7.5	171
17. R. S.	60	13.2	–	35.7	3.8	116
18. G. E.	75	14.5	–	31.6	4.2	112
19. E. DeP.	71	14.3	–	29.1	3.8	108
20. J. T.	62	13.5	–	37.8	3.7	254

*Determination not made because of elevated (49 mg. per cent) NPN.

Three of 5 pernicious anemia patients in relapse had normal values for blood and urine xylose and serum carotene. Diminished urinary excretion of xylose was noted in the remaining 2 (Cases 1 and 2, Table III), but increased as the hematocrit rose on vitamin B_{12} therapy. Blood xylose levels were normal with the exception of the initial value in Case 1. Serum carotene was initially depressed in these same 2 patients, but rose to low normal levels with treatment. Two of 3 patients had normal salivary iodide excretion times. The third had a delayed iodide appearance time which returned to normal on B_{12} therapy.

TABLE III. SERUM CAROTENE AND D-XYLOSE DETERMINATIONS IN PERNICIOUS ANEMIA PATIENTS IN RELAPSE

CASE	AGE	HEMATOCRIT	DATE	2-HOUR BLOOD XYLOSE (MG. PER CENT)	5-HOUR URINE XYLOSE (GM.)	SERUM CAROTENE (μG PER CENT)
1. J. H.	76	30	11/ 9/57	15.5	1.9	19.7
J. H.		38	11/18/57	39.7	0.4	36
J. H.		42	3/ 6/58	21.4	2.6	69
2. J. S.	75	30	1/ 3/58	32.2	0.8	27.5
J. S.		25	1/16/58	23.3	2.0	28.4
J. S.		42	3/ 6/58	40.0	4.1	45
3. M. H.	56	29	2/19/58	42.3	6.0	97
M. H.		37	3/16/58	42.1	6.0	115
4. M. C.	60	26	5/16/58	38.2	6.1	66
5. E. C.	72	26	5/15/58	43.1	4.2	82

Blood xylose levels in 6 patients with nonmegaloblastic anemia were normal (Table IV and Fig. 2). Urine xylose excretion was normal in 4 of this

group and low in the remaining 2 (Table IV and Fig. 1). Serum carotene was within the normal range in 5 patients. One subject with Cooley's anemia had a value of 40 μg per cent.

TABLE IV. SERUM CAROTENE AND D-XYLOSE DETERMINATIONS IN PATIENTS WITH ANEMIA OTHER THAN PERNICIOUS ANEMIA

CASE	AGE	HEMATOCRIT	DIAGNOSIS	2-HOUR BLOOD XYLOSE (MG. PER CENT)	5-HOUR URINE XYLOSE (GM.)	SERUM CAROTENE (μG PER CENT)
1. A. P.	41	18	D.U.* with acute and chronic blood loss	14.2	2.2	72
2. A. T.	35	25	Cooley's anemia	22.5	5.4	40
3. J. E.	74	27	Gastric polyp with chronic blood loss	42.5	4.5	75
4. G. M.	19	30	Hepatosplenomegaly with normocytic anemia	18.5	7.0	59
5. B. G.	18	35	Chronic infection with normocytic anemia	43.8	5.8	56
6. E. D.	43	35	D.U.* with chronic blood loss	57.1	2.9	181

*Duodenal ulcer.

DISCUSSION

Fishberg and Friedfeld[15] in 1932 demonstrated diminished urinary excretion of D-xylose in the presence of renal insufficiency. Helmer and Fouts[14] evaluating small intestinal absorption, noted a marked decrease in urine xylose excretion over a 5-hour period in 3 patients with steatorrhea. The importance of determining blood xylose was emphasized, in order to help differentiate diminished intestinal absorption from impaired renal clearance. Fourmann[8] and Benson and associates[9] also reported low 5-hour urinary xylose values (1 to 2 Gm.) in the sprue syndrome. Although some xylose is actively metabolized in the human body,[13, 16] the test is considered useful clinically in screening patients for intestinal malabsorption.

Although Helmer and Fouts[14] found slightly impaired absorption of xylose in a few of their patients with pernicious anemia in remission, no consistent abnormality was observed. Kidney function, as measured by urea clearance, markedly influenced the amount of xylose in the urine. In individual cases, however, urine xylose excretion did not necessarily parallel the urea clearance. Of our 20 patients in remission, 13 presented normal values for blood and urine xylose. The remaining 7 excreted diminished amounts of xylose in the urine. The presence of an elevated nonprotein nitrogen in Case 1 and normal blood xylose levels in Cases 2 to 7 favor diminished renal excretion rather than impaired intestinal absorption, although a minimal degree of the latter cannot be ruled out.

When patients in relapse were treated with specific antianemic therapy by Helmer and Fouts,[14, 17] there was a rise in urea clearance and an increase in xylose excretion. We also found increasing urinary xylose excretion in

2 of our 5 patients undergoing hematologic remission. The 3 remaining patients, despite a comparable degree of anemia, had normal urinary xylose values.

There was no consistent impairment of xylose absorption in patients with a moderate degree of anemia other than pernicious anemia. The presence of an elevated blood xylose level with low urinary excretion in Case 6 suggests impaired renal clearance. The possibility that a profound degree of anemia may result in impaired xylose absorption is raised by Case 1, who, with a hematocrit of 18 per cent, had low levels of both blood and urine xylose.

Wenger and associates[7] have shown by fat balance studies that diminished intestinal fat absorption correlates well with low serum carotene levels, values below 50 μg per cent being the rule. Elevated serum carotene may occur with dietary excesses of carotene-containing foods, and is also found in hypothyroidism, diabetes mellitus, and hyperlipemia. Low levels are seen with deficient intake, fever, jaundice, celiac disease, sprue, and hyperthyroidism[7, 21] Rubegni,[19] in a study of 5 patients with pernicious anemia, reported serum carotene levels within the lower limits of normal unrelated to the hemoglobin concentration.

In the present study, all pernicious anemia patients in remission had normal blood carotene levels. Three patients in relapse, with adequate dietary intake, presented low normal values. The remaining 2 patients with a history of anorexia, nausea, and vomiting showed low levels of serum carotene which rose progressively with therapeutic remission and resumption of normal food intake. Thus, reduced intake rather than impaired absorption would appear to explain these low values since it has been shown[18, 20, 22] that diet, the only source of body carotene, directly influences blood levels.

Five of 6 patients with anemia other than megaloblastic had carotene values within the normal range.

Excretion of salivary iodide following oral ingestion of potassium iodide was normal in 12 patients with pernicious anemia in remission in this study, whereas only 1 of 3 patients in relapse had a delay in excretion. These observations are at variance with those of Heath and Fullerton[10] who reported some delay in iodide excretion in 7 of 14 patients with pernicious anemia in remission. These differences may possibly be accounted for by variations in gastric emptying time, not measured in either study.

Clark[23] has shown that there is no abnormality of folic acid absorption in pernicious anemia.

SUMMARY AND CONCLUSIONS

No consistent impairment of intestinal absorption of D-xylose, carotene, or potassium iodide was demonstrated in patients with pernicious anemia in hematologic remission. Two of 5 patients in relapse showed low serum carotene levels and low levels of urinary xylose excretion in the presence of normal blood values. The increase in urine xylose content following therapy was

presumed to be due, in part, to increased renal clearance associated with a rising hematocrit. Resumption of normal eating habits was associated with a rise of serum carotene to low normal levels.

Although the possibility of minimal malabsorption of substances other than vitamin B_{12} exists in patients with pernicious anemia, particularly during relapse, the degree of malabsorption does not appear to be significant.

We wish to thank Dr. Bernard Cooper of the Thorndike Memorial Laboratory, Boston City Hospital, for his assistance in selecting the patients for this study.

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STUDIES IN LEUKEMIA

IX. The Induction of Leukemia in Adult C3H Mice by Means of Cell-Free Brain Filtrates

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THE opportunity to extend our studies[1-5] on the induction of leukemia with cell-free brain filtrates presented itself when a spontaneous leukemia developed in a 22-week-old C3Heb male mouse in our breeding colony.

MATERIALS AND METHODS

Preparation of Cell-Free Filtrates.—The cell-free filtrates of brain are prepared in the following manner:

1. Half of the mouse brain is removed immediately after death and placed in sterile saline solution in a 10 by 75 mm. test tube. When the brain is not to be processed immediately the tube is stoppered with a cotton plug and stored at −15° C.

2. Mortar and pestle for grinding, a 100 ml. graduated cylinder, and a flask are kept cold in the freezer.

3. An ice bath consisting of a pan filled with ice cubes and ice water is kept at 4° C. along with a flask of buffered isotonic saline solution of pH 7.4.

4. The cold mortar is placed in the ice bath. The brain is ground in the mortar with a small amount of saline solution and some sand. This is done in a cold room or in the refrigerator. It takes about 10 minutes until only a fine suspension remains, which is poured into the graduate; the mortar is rinsed with the cold buffered saline solution in order to dilute the brain suspension to 100 ml.

5. The suspension is then transferred to a flask where it is left to stand in the refrigerator for 24 hours. This step does not seem critical, however, because many filtrates have been used after settling for only an hour.

6. If after standing the supernatant is relatively clear, it may be decanted. If the supernatant is very cloudy, it is centrifuged at 3,000 r.p.m. for 15 minutes at 4° C.

7. Just before filtration the supernatant is contaminated with *Esch. coli*.

8. The contaminated supernatant is filtered through a Seitz so-called sterilizing pad (Type S).

9. A sample specimen is taken for bacterial culture before and after filtration.

10. Either the filtrate is used immediately or it is stored until needed in sterile cotton-plugged tubes at −15° C.

11. For intraperitoneal inoculations, 0.5 ml. of the filtrate is used. For intracerebral inoculations, from 0.075 to 0.1 ml. is used.

12. Recipients are usually 4- to 12-week-old mice.

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Preparation of Tumor Cell Suspensions.—Tumor cell suspensions are prepared by homogenizing tumor in a glass tissue mill and diluting the cells with physiologic saline solution to a concentration of approximately 10^6 per cubic centimeter. Tumor cell inoculations are made intraperitoneally with 0.5 ml. of the cell suspension.

Source of Mice.—The C3Heb mice were obtained either directly from Jackson Memorial Laboratory or were bred from a pedigreed stock originally obtained from them. The C3H mice were from Jackson Memorial Laboratory or from Cumberland View Farm, Clinton, Tenn. The C3H (Heston) mice were obtained from Dr. Sarah Stewart, National Institute of Health. The C3H x 101 hybrid mice were from Cumberland View Farm, Clinton, Tenn. The C3H (Rockland) mice were obtained from Rockland Farms, New City, N. Y.

RESULTS

Characteristics of Spontaneous Leukemia.—The 22-week-old male C3Heb mouse which served as the springboard for these experiments was 1 of 12 C3Heb mice in which spontaneous leukemia developed in our colony. Nine of the 12 were female, 3 were male. Their ages ranged from 22 to 91 weeks (average 50.4 weeks). Four of the animals were closely related. One female and one male were litter mates; the female had 3 litters; 2 of the mice of the third litter also developed leukemia. None of the female progeny had subsequent litters; thus, this line could not be studied further.

The spontaneous leukemias were characterized by a generalized lymphadenopathy, including a discrete mesenteric tumor, enlargement of the thymus, and moderate hepatosplenomegaly. The blood and marrow were studied in 4 of the spontaneous leukemias. Of the white cells in the blood, from 5 to 95 per cent were primitive lymphoid cells. When the percentage was low, the lymphocytes were increased in number; many showed bilobed and bizarrely-shaped nuclei; the cytoplasm frequently took a deep basophilic strain. The marrow was partly or completely replaced by the lymphoid cells. These varied in size from 12 to 16 micra and had a deep basophilic cytoplasm. The nuclei showed distinct pleomorphism and usually occupied about four-fifths of the cell; the chromatin was coarse and one or more nucleoli were frequently present.

The histologic changes in these animals resembled those observed in the lymphoblastoma of AKR mice.

Attempts were made to pass two of the other spontaneous leukemias with both tumor cell suspensions and brain filtrates. Those attempts were unsuccessful in 8 C3H and 31 C3Heb mice.

There were a total of 1,538 C3Heb mice over 22 weeks of age in our breeding colony which had never been used for experimental purposes. Those included 827 females and 711 males. This gives an incidence of 1.1 per cent in females and 0.4 per cent in males, for an over-all incidence of 0.6 per cent. This is similar to the incidence of 3 in 545 (0.6 per cent) females and 1 in 273 (0.4 per cent) males in Dr. Elizabeth Fekete's studies at Jackson Memorial Laboratory.[4]

Transmission by Means of Tumor Cell Suspensions.—The data for the transmission of the C3H lymphoma by means of tumor cell suspensions are summarized in Table I.

TABLE I. RESULTS OF INTRAPERITONEAL INOCULATION OF C3H MICE WITH TUMOR CELL SUSPENSIONS

GROUP	RECIPIENT STRAINS	SEX	RANGE OF SURVIVAL (DAYS)	NO. EXPERIMENTS	NO. LEUKEMIC / NO. INJECTED	PER CENT LEUKEMIC
1	C3Heb (Jax)	F	10-17	7	39/40	97
2	C3Heb (Jax)	M	9-21	10	56/59	95
3	C3H (Jax)	F	10-18*	7	31/31†	100
4	C3H (Jax)	M	13-19	3	22/25	88
5	C3H (Clinton)	F	10-15	6	37/39	95
6	C3H (Clinton)	M	13-15	1	9/10	90
7	C3H (Heston)	F	9-14	5	31/35	88
8	C3H (Heston)	M	10-22	3	14/14	100
9	C3H (Rockland)	F	17-21	2	4/20	20
10	C3H (Rockland)	M	16-21	1	4/10	40
11	C3H x 101 (Clinton)	F	13-18	4	19/24	79
12	C3H x 101 (Clinton)	M	14-20	4	27/34	79
				53	293/341	86

*One animal survived 35 days.
†Five animals (in one experiment) that died of infection within the first 3 days after inoculation are not included.

1. In Group 7 all animals succumbed to the transplanted lymphoblastoma in 4 of the experimental groups. In one group, all 4 animals survived the tumor cell inoculation.

2. In Groups 11 and 12, 12 mice died of a salmonella infection. In all others lymphoblastoma developed.

3. Significant differences are not apparent between the susceptibility of the sexes, or the substrains, with the exception of the C3H (Rockland) mice (Groups 9 and 10). Although only a few experiments with these animals have been done, it appears that their susceptibilty to this tumor is far less than that of the other substrains.

4. Data do not indicate any significant difference between isologous and homologous passage of the tumor. Of the 53 experiments, 21 were made within the substrain, and 32 were across substrain lines. Neither the incidence nor the latency is influenced by the source of the tumor, or by continuous passage. One tumor line has been maintained through 14 passages. Leukemia developed in 9 out of 10 mice in 17 to 20 days in the first passage, and developed in 10 out of 10 in 13 to 18 days in the fourteenth passage.

Transmission by Means of Cell-Free Brain Filtrates.—The data for the transmission of the C3H lymphoma by cell-free brain filtrates are summarized in Table II.

1. There is no significant difference in either incidence or latency with regard to the sex of the recipient.

2. The low incidence of leukemia in Group 3 is accounted for in part by the fact that 6 animals of one group died within 48 hours after injection. Interestingly, however, in none of the 5 mice of the other group in this same experiment did leukemia develop when the filtrate of a C3H (Jax) brain was used

for filtrate source. This same filtrate failed to produce leukemia in any of 10 C3Heb (Jax) mice when inoculated intraperitoneally (Group 5). In one other experiment, a filtrate prepared from the brain of a C3H (Heston) mouse failed to induce leukemia in any of 5 female C3H (Heston) mice inoculated intraperitoneally (Group 13).

TABLE II. RESULTS OF INOCULATION OF C3H MICE WITH CELL-FREE BRAIN FILTRATES

GROUP	RECIPIENT STRAINS	SEX	ROUTE INOCUL.	RANGE (DAYS) SURVIVAL	NO. EXPERIMENTS	NO. LEUKEMIC / NO. INJECTED	PER CENT LEUKEMIC
1	C3Heb (Hektoen)	F	I.C.	14-29	3	12/13	92
2	C3Heb (Hektoen)	M	I.C.	14-29	3	16/16	100
3	C3Heb (Jax)	F	I.C.	13-17	2	4/15*	27
4	C3Heb (Jax)	M	I.C.	14-25	5	39/42	93
5	C3Heb (Jax)	F	I.P.	9-17	4	23/34	67
6	C3Heb (Jax)	M	I.P.	14-20	1	9/10	90
7	C3H (Jax)	F	I.C.	12-20	4	29/29	100
8	C3H (Jax)	F	I.P.	13-28	3	15/15	100
9	C3H (Jax)	M	I.P.	22-27	1	5/5	100
10	C3H (Clinton)	F	I.C.	12-18	1	14/15	93
11	C3H (Clinton)	F	I.P.	10-15	2	23/25	92
12	C3H (Clinton)	M	I.P.	12-21	3	38/40	95
13	C3H (Heston)	F	I.P.	11-15	3	9/14	65
14	C3H (Heston)	M	I.P.	10-19	2	9/9	100
15	C3H (Rockland)	F	I.P.	38-46	1	2/10	20
16	C3H (Rockland)	M	I.P.	10-37	2	11/20	55
17	C3H x 101 (Clinton)	F	I.P.	13-29	5	35/39	89
18	C3H x 101 (Clinton)	M	I.P.	13-29	3	16/27	60
					48	309/378	82

I.C. = intracerebral.
I.P. = intraperitoneal.
*Six animals that died within 48 hours of inoculation are included.

As in the passages with tumor cell suspensions, the significantly lower incidence in C3H (Rockland) mice (Groups 15 and 16) should be observed. The latent period for the development of leukemia in C3H (Rockland) females also seems longer than in any of the other mice, and although the males of this strain also show some delay in the development of leukemia, the degree is much less striking.

3. Significant differences were not demonstrable in the results regardless of isologous or homologous brain filtrates. Of the 48 experiments, 27 were within the substrains and 21 were across substrains. Continuous passages, up to 5, did not influence either the latency or incidence of the development of leukemia. In 15 of the 48 experiments the filtrates were prepared from the brains of mice in which leukemia developed as the result of the inoculation with tumor cell suspensions. Of the 141 mice inoculated with these filtrates, leukemia developed in 120 (85 per cent). This was similar to the incidence in the other 33 experiments, indicating that there is no significant difference between the various sources of the brain filtrates regarding their ability to produce leukemia.

4. In addition to the animals shown in Table II, 14 animals were injected subcutaneously with cell-free filtrates of brain. Ten of these were less than 24 hours old at the time of injection. In all 10 of these mice subcutaneous tumors developed at the injection site (Fig. 1); moreover, disseminated disease occurred, and the animals died in from 20 to 50 days after injection. The remaining 4 animals were 1 week of age at the time of inoculation. In 2, leukemia developed in 20 and 26 days.

Fig. 1.—Fifty-day-old C3Heb mouse injected as newborn with cell-free brain filtrate. Massive subcutaneous lymphosarcoma at site of injection.

5. Twenty mice were injected with a cell-free brain filtrate which had been exposed to 100,000 r delivered by the Telecobalt^{60} machine. Leukemia developed in 11 of these mice.

TABLE III. RESULTS OF INOCULATION OF C3H MICE WITH HEAT-INACTIVATED BRAIN FILTRATES

SOURCE OF FILTRATE	RECIPIENT STRAINS	ROUTE OF INJECTION	UNHEATED NO. LEUKEMIC / NO. INJECTED	HEATED NO. LEUKEMIC / NO. INJECTED
C3H (Jax)	C3H (Jax)	I.P.	15/15	0/10
C3H (Jax)	C3H (Jax)	I.P.	15/15	0/10
C3Heb (Jax)	C3H (Clinton)	I.C.	14/15	0/8
C3H (Clinton)	C3H (Clinton)	I.P.	10/10	0/10

The Effect of Inoculation of Control Brain Filtrates Into C3H Mice.—The results with heat-inactivated (65° C. for 45 min.) leukemic brain filtrates are summarized in Table III. Female mice were used in these experiments. It is apparent that heat inactivates the filtrates.

The results of inoculating C3H mice with cell-free brain filtrates prepared from nonleukemic C3H mice are summarized in Table IV. Leukemia did not develop in any of the mice so treated.

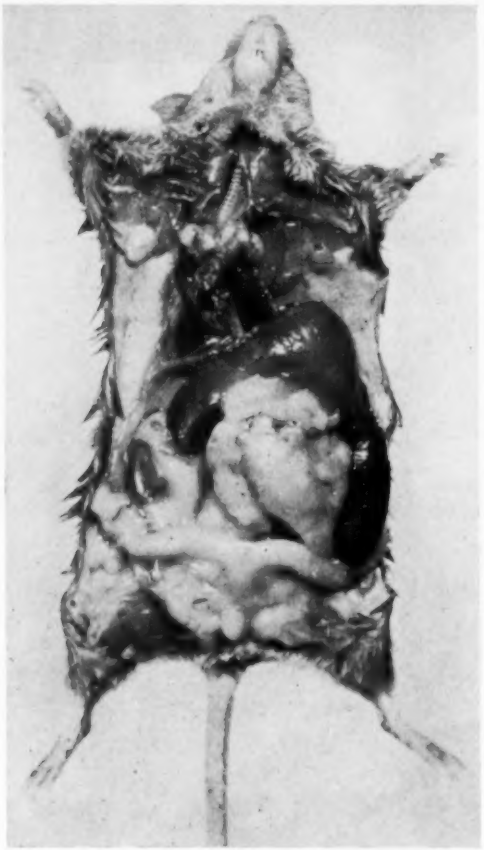

Fig. 2.—Adult C3H mouse, 14 days after intracerebral inoculation of cell-free brain filtrate. Note massive diffuse mesenteric involvement with invasion of kidneys and genital organs.

Pathologic Changes.—The gross appearance of the induced leukemia is illustrated in Fig. 2. The characteristic is a massive, diffuse infiltration of the mesentery with involvement of the urogenital organs, the involvement frequently extending through the peritoneum to infiltrate the abdominal and

perispinal musculature. Involvement of the liver and spleen varies from slight to massive. Peripheral lymph node involvement is usually only moderate, and thymic involvement is modest.

TABLE IV. RESULTS OF INOCULATION OF C3H MICE WITH NONLEUKEMIC BRAIN FILTRATES

SOURCE OF FILTRATE	RECIPIENT STRAIN	SEX	ROUTE OF INJECTION	NO. EXPERIMENT	NO. LEUKEMIC / NO. INJECTED
C3H (Jax)	C3H (Jax)	F	I.P.	2	0/16
C3H (Jax)	C3H (Clinton)	M	I.P.	2	0/15
C3Heb (Jax)	C3H (Clinton)	F	I.C.	2	0/15
C3H (Clinton)	C3H (Clinton)	F	I.P.	1	0/10

The histologic changes are typical of mouse lymphoblastoma and are indistinguishable from those described previously regarding Swiss mice.[3]

Among the 25 animals whose blood was studied, the presence in 9 of at least 5 per cent primitive lymphoid cells was considered diagnostic of "leukemia." Whenever the blood was considered diagnostic, the marrow was always frankly involved. Of the 6 animals whose blood was not considered diagnostically characteristic, diffuse or massive infiltration by tumor cells was present in the marrow.

DISCUSSION

The induction of leukemia in C3H mice by means of cell-free brain filtrates adds further evidence to the thesis that leukemia may be induced by an agent present in cell-free extracts of brain. The characteristics of this agent with regard to resistance to roentgen ray, heat inactivation, and filterability are comparable to those previously described for the filtrates prepared from the brains of AKR[1] and Swiss[3] mice and from the brains of patients dying of acute leukemia.[2]

As with the other filtrates, the percentage of animals in which leukemia developed after inoculation is high, and tends to approach an all-or-none response. Occasionally, leukemia fails to develop in whole groups of animals when inoculated with a brain filtrate. Resistance in these animals represents a phenomenon different from that observed in C3H (Rockland) mice in which the incidence of leukemia is low as induced with both tumor and filtrate. In the case of filtrate-induced leukemia, the substrain seems to have a specific resistance to the leukemia. In tumor-induced leukemia, there is no resistance to tumor cell inoculation; resistance to filtrate inoculation usually occurs in whole groups of animals inoculated with the same filtrate. For this reason it appears that these negative experiments result from a function of the filtrate rather than of the host.

The induction of leukemia by a cell-free brain filtrate compels the question regarding the mechanics of leukemogenesis. Pathogenic studies[5] demonstrate the multicentric origin of this disease. They suggest that the pluripotential mesenchymal cells are capable of undergoing leukemic transformation anywhere in the body.

We believe that the cell-free brain filtrate is rich in the virus which, whether injected intracerebrally or intraperitoneally, becomes rapidly disseminated, and begins to multiply. The mesenchymal cell proliferation characteristic of this virus multiplication is a defensive host reaction. In the newborn animal, the more primitive and energetic mesenchymal host reaction is capable of producing subcutaneous tumors at the site of injection in addition to producing a disseminated disease.

SUMMARY

1. Spontaneous leukemia developed in a 22-week-old male C3Heb mouse.

2. Tumor cell suspensions obtained from the original leukemic mouse or from other mice to which leukemia was transmitted when injected into various C3H substrains and C3H x 101 hybrids caused leukemia in 293 of 341 mice (86 per cent).

3. Cell-free brain filtrates, similarly obtained, inoculated into the same substrains induced leukemia in 309 of 373 mice (82 per cent).

4. The experiments further substantiate the thesis that leukemia may be induced in the mouse by a cell-free agent having the characteristics of a virus.

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SEROLOGIC REACTIONS IN ADENOVIRUS DISEASE

I. Delayed Complement-Fixing Antibody Response

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DURING the past several years there has been steadily increasing evidence to implicate certain members of the adenovirus family as important in the causation of respiratory disease in military populations. The syndromes with which the implicated virus types have been associated cover a spectrum that ranges from relatively simple upper respiratory infections to atypical pneumonias of varying severity.[1-6]

Laboratory techniques applicable to the etiologic diagnosis of adenovirus disease include virus isolation, neutralization tests, and complement fixation tests.[1-2, 6-7] The first two methods are quite cumbersome and even the colorimetric modification of the neutralization test described by Lennette and associates[8] is relatively time consuming and must be rigidly controlled. Furthermore, isolation of an adenovirus from a patient does not necessarily prove its causal relationship to the illness.

Complement fixation tests, on the other hand, afford a fairly simple means of detecting adenovirus infections, and the relative simplicity of the technique permits its application to fairly large groups of individuals. Although the method does not identify the adenovirus serotype responsible for the infection, the technique can be utilized with a considerable degree of accuracy in epidemiologic studies, particularly when ancillary knowledge is available as to the serotypes that are operating in the population under study. In serologic tests for the diagnosis of viral infections, and including those for the adenoviruses, a diagnostically significant finding is considered to be the occurrence of a fourfold or greater rise in antibody titer during the course of the illness. The detection of increases in antibody is based on the examination of multiple serum specimens. The first specimen is obtained early in the course of the illness, and the second specimen is usually obtained about two weeks after the first. It has been our experience that this generalization is applicable to the study of adenovirus infections, but exceptions have been encountered and form the basis of the present report. In this connection it is noteworthy that

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the patient from whom the prototype RI-67 strain of type 4 adenovirus was isolated did not show a rise in complement-fixing antibody level at 14 days after onset of illness, but a rise in antibody titer was detected at a later time.[1]

MATERIALS AND METHODS

Collection of Serum Specimens and Throat Washings.—The blood samples and throat washings examined in this study were collected in the course of a more comprehensive study of respiratory disease conducted at Fort Ord, Calif., from September, 1954, through August, 1955. A part of that major study was concerned with the proportion of patients admitted to the Station Hospital with respiratory disease attributable to infection with an adenovirus.

For purposes extraneous to this report, 3 blood samples were obtained wherever feasible from men admitted to the respiratory disease wards of the Station Hospital. Feasibility depended on the individual's remaining on-post sufficiently long to permit collection of the second and third blood samples. The first specimen (acute phase) was obtained as soon as possible after the onset of illness; the second (convalescent phase) was obtained, insofar as possible, 14 days thereafter; and the third specimen (late convalescent phase) was taken 14 or more days after the second.

Throat washings were not obtained from every patient because of the technical burden posed by the collection and testing of such a huge volume of material. Instead, washings were collected from a cross-section of the population, namely, from 434 of the 3,060 patients admitted to the study; the specimens were obtained at the time the first blood sample was taken.

Laboratory Tests Employed.—These consisted of the complement fixation and neutralization tests and virus isolations.

Complement fixation tests were done according to the technique employed in this laboratory for some years and described in a recent compendium on diagnostic methodology.[9] The antigen consisted of HeLa cell cultures infected with the RI-67 strain of type 4 adenovirus. Sets of serum samples (first and second, or first and third specimens) from the same individual were always examined simultaneously in the same tests.

Neutralization tests were done by the colorimetric technique described in another communication from this laboratory.[8] Acute and late convalescent phase serum specimens from the same individual were always examined simultaneously in the same test. Three adenovirus serotoypes, viz., 3, 4, and 7, were used. The type 3 strain was the GB strain, the type 4 was the RI-67 strain, and the type 7 was the Gomen strain.

Virus isolation was based on the use of HeLa cell culture systems. Identification and typing of cytopathogenic agents as adenoviruses were done by the colorimetric method,[8] and monotypic antisera (rabbit) were employed.

RESULTS

During the period of this study, adenoviruses were recovered from 129 of the 434 individuals from whom throat washings were collected and tested. Subsequent typing of these 129 adenoviruses showed the following distribution of serotypes: 1 of type 3, 85 of type 4, and 43 of type 7.

The multiple serum specimens from those individuals from whom an adenovirus had been isolated were examined for their complement-fixing and neutralizing antibody levels, and the findings evaluated against virus isolation as a base line.

Delay in the Appearance of Complement-Fixing Antibody.—Collation of the results of the complement fixation tests with the results of virus isolation

tests revealed that in 120 of the 129 patients from whom an adenovirus was isolated, a fourfold or greater rise in antibody titer occurred during the approximate 2-week interval between the drawing of the acute phase blood specimen and the early convalescent phase blood specimen. Stated in other terms, a diagnostically significant rise in complement-fixing antibody titer was encountered in 93 per cent of the patients from whom an adenovirus was recovered. The remaining 9 individuals from whom an adenovirus had been isolated showed no rise in complement-fixing antibody titer in the interval between the acute and early convalescent phase bleedings. The findings on these 9 patients are presented in Table I. It will be noted that the acute phase blood specimens had been obtained within 5 days of the onset of illness, and that the early convalescent phase specimens had been obtained from 14 to 20 days after onset. The absence of antibody at these relatively late periods after onset of illness bore no obvious relation to the serotype of the virus recovered, inasmuch as 1 patient yielded a type 3 virus, 5 patients yielded a type 4, and 3 patients yielded a type 7 virus. These cases were not restricted to any one temporal period, but occurred throughout the study year and were intermingled with a much larger number of patients from whom an adenovirus was isolated and who showed diagnostically significant rises in complement-fixing antibody titer.

TABLE I. ABSENCE, OR DELAYED APPEARANCE, OF COMPLEMENT-FIXING ANTIBODY IN ADENOVIRUS DISEASE

SUBJECT NUMBER	VIRUS ISOLATED (SEROTYPE)	ACUTE PHASE BLOOD		CONVALESCENT PHASE BLOOD		LATE CONVALESCENT PHASE BLOOD	
		DAYS FROM ONSET	C. F. TITER	DAYS FROM ONSET	C. F. TITER	DAYS FROM ONSET	C. F. TITER
1	3	3	<1:8 <1:8	18	<1:8	31	1:64
2	4	4	<1:8 1:8	17	<1:8	43	1:256
3	4	3	<1:8 <1:8	14	<1:8	33	1:32
4	4	4	<1:8 <1:8	18	<1:8	38	<1:8
5	4	3	<1:8 1:8*	17	1:8	37	1:64
6	4	4	<1:8 <1:8	20	1:8	36	1:32
7	7	1	<1:8 <1:8	15	<1:8	29	1:16
8	7	5	<1:8 <1:8	19	<1:8	36	<1:8
9	7	2	<1:8 <1:8	18	<1:8	49	1:16

*Nonspecific fixation.

The tests on these serologically negative patients were repeated using the acute phase blood specimen and a third, or late convalescent, specimen; the latter specimens had been taken from 29 to 49 days after the onset of the respiratory illness (Table I). As is shown in Table I, in 7 of the 9 patients a significant rise in complement-fixing antibody had occurred at some time during the interval between the second and third bleedings, i.e., between the time

the early convalescent and the late convalescent blood specimens had been taken. In two instances (Subjects 4 and 8), the isolation of an adenovirus was not accompanied by the appearance of detectable amounts of complement-fixing antibody, even with blood specimens taken as late as 36 and 38 days after the onset of the illness.

Relationship Between Virus Isolation and Neutralizing Antibody Titers.—To determine whether the situation with respect to neutralizing antibody paralleled or differed from that observed with the complement-fixing antibody, neutralizing antibody levels were determined in the sera of those 9 individuals described above and listed in Table I. Unfortunately, by this time the early convalescent phase (2 to 3 weeks) serum specimens were no longer available, so that antibody determinations were performed on the acute phase and late convalescent phase blood specimens. As is shown in Table II, a four-fold or greater rise in neutralizing antibody level was demonstrable in all 9 individuals, including the 2 patients in whom no detectable complement-fixing antibody response was observed.

TABLE II. NEUTRALIZING ANTIBODY RESPONSE IN PATIENTS IN WHOM COMPLEMENT-FIXING ANTIBODY RESPONSE TO ADENOVIRUS INFECTION WAS DELAYED OR NONDEMONSTRABLE

SUBJECT NUMBER	VIRUS ISOLATED (SEROTYPE)	ANTIBODY TITER TO TYPE 3 VIRUS: ACUTE PHASE BLOOD	TYPE 3 VIRUS: LATE CONV. PHASE BLOOD	TYPE 4 VIRUS: ACUTE PHASE BLOOD	TYPE 4 VIRUS: LATE CONV. PHASE BLOOD	TYPE 7 VIRUS: ACUTE PHASE BLOOD	TYPE 7 VIRUS: LATE CONV. PHASE BLOOD
1	3	<1:4	1:16	<1:4	<1:4	<1:4	<1:4
2	4	<1:4	<1:4	<1:4	1:16	<1:4	<1:4
3	4	<1:4	<1:4	<1:4	1:32	<1:4	<1:4
4	4	<1:4	1:16	<1:4	1:8	<1:4	1:8
5	4	<1:4	1:8	<1:4	1:16	<1:4	<1:4
6	4	1:4	1:4	<1:4	1:16	<1:4	<1:4
7	7	<1:4	1:8	<1:4	1:16	<1:4	1:4
8	7	<1:4	1:32	<1:4	1:8	<1:4	<1:4
9	7	<1:4	1:4	<1:4	1:16	<1:4	<1:4

A point of interest was the heterotypic nature of the neutralizing antibody response. In 4 patients (Subjects 1, 2, 3, and 6) the neutralizing antibody response was homotypic, and there was no evidence of an antibody response to a heterotype. In 4 other individuals (Subjects 4, 7, 8, and 9), the heterotypic antibody response was greater than the response to the patients' own infecting virus, i.e., the homotypic response. In a fifth patient (Subject 5), who showed a rise in heterotypic antibody, the rise was of a slightly lesser magnitude than the rise in homotypic antibody. A surprising feature was the failure of all 3 individuals from whom a type 7 virus was isolated to develop neutralizing antibody to this serotype; all 3 individuals responded, rather, with significant increases in type 4 antibody, and 2 of the 3 also showed diagnostically significant increases in type 3 antibody levels. The number of individuals (4 of 8) who responded heterotypically with type 3 antibody is quite high and appears disproportionate in view of the fact that a type 3 virus was

encountered only once among 129 adenovirus isolations. It is also of interest that the greatest heterotypic increases in type 3 neutralizing antibody occurred in the 2 patients (4 and 8) who failed to show demonstrable amounts of complement-fixing antibody in their blood even one month after the onset of illness.

DISCUSSION

The data presented do not impugn the validity of studies using complement fixation tests as the primary tool in the diagnosis of adenovirus disease in adults. The lack of correlation between the isolation of an adenovirus and the appearance of a complement-fixing antibody response within 2 to 3 weeks after onset of illness occurred in only 5 per cent of the 129 cases. Under some circumstances, however, such discrepancies might assume some importance. It should be pointed out, however, that the results reported in this paper were derived from studies during one year on a single, rather homogeneous population of young men; thus, it is not inconceivable that under some circumstances, as in a population group not previously exposed to infection, a greater lack of correlation might be encountered.

Prior to the completion of this report, the paper of Katz and associates[10] described a disassociation between neutralizing and complement-fixing antibody levels. Their studies dealt with adenovirus types 1 to 4, and were concerned with individuals who could not be considered to be suffering from adenovirus disease, but who had residual antibody. They comment that the lack of consistent relationship between neutralizing and complement-fixing antibody deserves further consideration in relation to its significance in adenovirus infections and adenovirus disease. From one point of view, namely, the practicality of using complement fixation tests in the serodiagnosis of adenovirus disease, the data presented herein afford a partial answer. Nonetheless, it must be remembered that this study was carried out in a circumscribed group and that the results might be quite different under other conditions. Differences might also be observed with other adenovirus types.

There is general agreement that adenovirus types 3, 4, and 7 are, *at present,* the most prominent causes of adenovirus disease in military populations in the United States (see précis in van der Veen and Kok[11]). It is becoming clearer that adenovirus disease due to type 3 virus is, at least currently, of the least proportionate importance. The limited body of data presented herein suggest there is a tendency for infections with type 4 or type 7 viruses to elicit a heterotypic antibody response to type 3 virus. This finding agrees with the observations of Hilleman and associates,[12] who noted that inoculation with vaccines containing only type 4 and type 7 adenoviruses called forth an appreciable rise in antibody to type 3 virus. Both observations thus afford additional evidence for the concept that disease due to type 3 virus is relatively less important, incidence-wise, in this age group (recruits) because of previous experience.

SUMMARY

In young men with respiratory disease there was usually good correspondence between the isolation of an adenovirus and the appearance of an increase in complement-fixing antibody in about 2 to 3 weeks. In 9 of the 129 cases, an increase in complement-fixing antibody in this period of time was not demonstrable, but in 7 of these 9 instances a definitive increase in antibody titer could be found after 29 or more days. In 2 of the 9 cases, complement-fixing antibody failed to rise even at this late time.

When the acute and late convalescent phase sera were assayed for neutralizing antibody, a rise in titer was detected in all 9 individuals in whom demonstrable complement-fixing antibody either failed to appear or was delayed in appearing. The neutralizing antibody response was often heterotypic, however, bearing no relationship to the virus serotype isolated from the individual.

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MIXTURES OF COAGULASE-POSITIVE STAPHYLOCOCCI AND STRAIN VARIATION IN SPECIMENS FROM CLINICAL SOURCES

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THE recognition of staphylococcal strains either as discrete in origin or as variants of a common parental strain has been greatly simplified in recent years by the development and adoption of bacteriophage typing methods.[1-3] The occurrence of mixed growth of staphylococci would be expected from the ubiquitous distribution and large numbers of known types. There is a common tendency, however, to treat clinical specimens as containing only a single strain of a given pathogen or potentially pathogenic species. In the course of routine bacteriophage typing of staphylococci it was learned that a specimen so treated and recorded as nontypable, actually proved to be a mixture of two typable strains with complementary patterns of phage sensitivity. This observation was expanded into the following study of the incidence of mixtures of coagulase-positive staphylococci in routine clinical specimens. Staphylococcus Medium 110 was found to markedly stimulate pigment development; pigment variation, therefore, was employed in the presumptive detection of mixtures, followed by confirmatory tests of bacteriophage typing and antibiotic sensitivity testing on the isolated strains.

METHODS

Routine clinical specimens received by the bacteriology laboratory at the Parkland Memorial Hospital were streaked on blood agar plates. Confluent growth from plates containing coagulase-positive staphylococci was transferred to trypticase soy agar slants for 4 to 6 hours at 37° C. for enrichment and then streaked on plates of Staphylococcus Medium 110. These were incubated 48 hours at 30° C. and an additional 48 to 72 hours at room temperature (25° C.). Plates were examined daily after the second day for mixtures differing in pigmentation. Only mixtures in which both components were coagulase-positive by the slide coagulase test were further examined for antibiotic sensitivities and typed with bacteriophages.

The bacteriophage typing methods of Blair and Carr[2] were employed for typing the strains with a set of 25 typing phages kindly supplied by Dr. Blair.

The resistance of each strain to 10 antibiotics and to triple sulfa was determined with the use of sensitivity disks (Baltimore Biological Laboratory).

RESULTS

A total of 196 clinical specimens containing coagulase-positive staphylococci was screened for mixtures based on pigmentation. Sixteen coagulase-positive mixtures were obtained, ranging from pigmented plus white strains, readily detected by the first inspection, to pigmented mixtures which did not differ in color intensity until the fourth day. Most of the pigmented mixtures would not have been detected on the original blood agar plates.

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TABLE I. MIXTURES OF COAGULASE-POSITIVE STAPHYLOCOCCI FROM CLINICAL SPECIMENS

SPECIMEN NO.	SOURCE	PIGMENT	PHAGE PATTERN	DIFFERENCES IN ANTIBIOTIC PATTERN*
1	Ear infection	Light orange Dark orange	47/53/77/VA4/42D 73	TC, AM, TM, SM
2	Throat swab	Light orange Dark orange	42D/44A 3A	PC
3	Meningitis	Light orange Dark orange	Nontypable Nontypable	
4	Postoperative infection	Light orange Dark orange	7/47/54/70/73/75/44A Nontypable	AM, EM
5	Tracheal aspiration	Light orange Dark orange	44A 44A	
6	Postoperative infection (†)	Orange White	Nontypable 52/52A/7/44A	SSS
7	Lung (autopsy)	Light orange Dark orange	7/47/53/54/73/75/77/VA4/44A Nontypable	AM, EM
8	Tracheal aspiration	Light orange Dark orange	44A 7/47/53/54/73/75	TC, AM, TM, PC, SM

9	Burn infection	Light orange Dark orange	47/53/VA4 Nontypable	AM, EM
10	Postoperative infection	Light orange Dark orange	79/7/47/53/54/73/77/VA4/44A 53	TC, AM, TM
11	Wound infection	Light orange Dark orange	77 Nontypable	TC, AM, TM SSS
12	Sputum	Orange White	Nontypable 44A	PC
13	Sputum	Orange White	Nontypable 44A	PC
14	Infected umbilicus	Light orange Dark orange	7/47/54/73/75 44A	TC, AM, TM, PC, SM, SSS
15	Throat swab	Orange White	3A/44A Nontypable	TC, AM, TM, BA, PC
15a	Sputum	Orange White	3A/44A Nontypable	TC, AM, TM, BA, PC
16	Ulcer	Light orange Dark orange	Nontypable Nontypable	TM

*Only differences are reported and symbol is placed on line with the resistant strain. TC = tetracycline; AM = chlortetracycline; TM = oxytetracycline; SM = streptomycin; EM = erythromycin; BA = bacitracin; SSS = triple sulfa; PC = penicillin.

Mixtures containing a coagulase-negative component occurred frequently but were not included.

Table I summarizes the results of bacteriophage typing and antibiotic sensitivity testing of the individual strains from the mixtures. Two specimens (3 and 5) probably represent color variants since the members of each mixture differ only in the property of pigmentation. All other specimens are presumably mixtures of two distinct strains, and all but one (16) are composed of either two bacteriophage types or a typable and nontypable strain. Since one-third of all the strains studied were nontypable, a mixture of nontypable strains would be expected; therefore, Strain 16 was considered a probable mixture on the basis of differences in antibiotic sensitivity and pigment. Two specimens (15 and 15a) are identical mixtures from the same patient and are regarded as one mixture. Mixtures of two coagulase-positive strains were therefore obtained from 14 individuals, or 7 per cent of the total number of specimens.

Several of the bacteriophage patterns are typical of the hospital strains of staphylococci and are probably hospital-acquired. In one individual (4) infection with the typable component is of a transient nature, since specimens taken before and after the mixture appeared were pure cultures of a nontypable strain. In another individual (3), however, strain 44A replaced a previously isolated nontypable strain.

All of the strains from the mixtures were sensitive to neomycin and carbomycin and resistant to polymyxin B. Variable results were obtained with the other antibiotics. For simplicity, only differences in sensitivity are recorded in the table, and it can be seen that in almost all mixtures one strain is generally more resistant than the other to one or more antibiotics.

TABLE II. VARIATION OBSERVED IN 31 CLINICAL SPECIMENS CONTAINING STAPHYLOCOCCI OF TYPE 80/81

PHAGE PATTERN	NUMBER OF SPECIMENS	
	PURE STRAINS	MIXTURES OF COLOR VARIANTS
A. 52/42B/81	5	
B. 52/42B/44A/81	20	2
C. Mixture of A and B		3
D. Mixture of 52/52A/42B/44A/81 and 52/52A/42B/81		1
Total	25	6

From these results it would seem that pigment variation within a strain is not common, having occurred in only two instances in the above series. However, we have recently observed that clinical specimens containing staphylococci with bacteriophage pattern 52/42B/81 (type 80/81[4]) included a number of light- and dark-pigmented variants. In a hospital outbreak caused by this type, a series of 31 specimens, primarily nasal swabs, were positive for the epidemic staphylococcal strain. Six of the specimens were mixtures of pigment variants and, of these, 4 also varied in bacteriophage typing pattern (Table II).

Variation in sensitivity to bacteriophage 44A has been previously reported for this staphylococcal type.[4, 5] The strain sensitive to bacteriophage 52A may differ from the type 80/81, although by the criteria of Williams and Rippon[1] they would be considered the same strain. There is some indication, however, that isolates with a typing pattern 52/52A/42B/81 are not as virulent as typical strains of type 80/81.[6]

Differences in antibiotic sensitivity were not observed within these mixtures containing type 80/81 strains, although some variation occurred between strains from different specimens. The degree of pigmentation did not correlate with sensitivity or resistance to phage 44A.

DISCUSSION

The observed 7 per cent incidence of mixed growth represents a lower limit since primary detection is based on a single property, pigmentation, and is not of sufficient sensitivity to detect mixtures in which one component occurred in proportions lower than 1 per cent. The ratios may also have been altered by the preliminary, but unavoidable, subculture. The method is sensitive enough, however, to detect mixtures not apparent on the original blood agar plates. The proportion of samples with mixed growth agrees closely with that observed by Bynoe and associates[7] but is considerably less than the 12 per cent observed by Wallmark.[8] When mixtures are not recognized as such and are regarded as pure strains, the laboratory findings may be misleading. Bacteriophage typing and antibiotic sensitivity testing of an isolated colony would give a different set of results than similar tests performed on the mixed growth. This may be of particular importance if the two strains differ in virulence and treatment is based on the laboratory findings for the strain of lesser virulence. The possibility of mixed infection should be considered in instances in which response to therapy does not coincide with laboratory findings of antibiotic sensitivity.

The lysogenic state, common in coagulase-positive staphylococci, undoubtedly influences the prevalence and stability of such mixtures, since phage carried by one strain may lyse or lysogenize the other. A superinfecting strain can thus have a transient existence, can replace the original strain, can interact to produce new types, or may coexist as a stable mixture.[9] Such interactions may be a factor in the variability of type 80/81 which has rapidly spread and colonized in hospitals throughout the world.[4, 10] If this type superinfects the nasal passages of a staphylococcus carrier, the interaction and lysogenization may produce the variation which we have observed. Another possibility is that type 80/81 has a higher spontaneous mutation rate than other strains of staphylococci. Variation in sensitivity to antibiotics reported from different localities for this type[4] might be evidence of genetic instability.

SUMMARY

1. Fourteen of 196 routine clinical specimens containing coagulase-positive staphylococci were shown to be mixtures of two strains.

2. The pairs of strains in these mixtures differed in pigmentation, sensitivity to one or more antibiotics, and in bacteriophage typing pattern.

3. Strains of bacteriophage type 80/81 showed a relatively high degree of variation in pigmentation and in bacteriophage typing pattern.

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ROLE OF ADRENAL HORMONES IN LYMPHOCYTE MOBILIZATION IN THE RAT

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IN A previous study we described a method for replacement-transfusion of rats with donor blood that had been rendered leukocyte free.[1] Rapid removal of blood was achieved by this technique, and the rate of leukocyte mobilization from storage areas for replenishment of the circulating blood leukocyte levels could be studied. The remarkable thing about these experiments was the extreme mobility of leukocyte reserves in the rat, so that total replenishment of the circulation occurred within 10 to 30 minutes after cessation of active leukocyte removal. Repeated periods of leukocyte removal failed to exhaust the reserves. Equally striking was the observation that both granulocytes and lymphocytes were brought back into the blood stream in quantities necessary to restore their original levels.

From these observations, we concluded that the rat has large pools of readily available, preformed leukocytes. Because of the rapidity with which these leukocyte reserves entered the blood stream, it seemed clear that they were in or near vessels from which they could enter the general circulation.

In the previous studies, we found no clue to factors that might control the magnitude or mobility of these leukocyte reserves. Many studies have indicated the important role of the adrenal hormones in the quantity of lymphoid tissue, and Dougherty and White[2] have emphasized the lymphopenic effects of adrenal hormones in rodents. Since the leukocytes of the rat are mostly lymphocytes, the present study was undertaken to determine what effects, if any hypophysectomy, adrenalectomy, or cortisone therapy would have on the readily mobilized reserves of lymphocytes in the rat.

METHODS

Sprague-Dawley or Sherman strain rats were used in all experiments. Heparinized donor rat blood was obtained by cardiac puncture, was centrifuged, and the buffy coat removed by the methods previously described.[2] The reconstituted donor blood regularly had leukocyte counts of less than 500 per mm.^{3}.

The replacement transfusions were performed using the previously described technique. Donor blood was injected into a femoral vein plastic cannula at a rate equal to the spontaneous outflow from a cannula in the femoral artery. Replacement transfusions of 20 ml., found to represent at least 80 per cent blood volume replacement,[1] were accomplished within 5 minutes or less.

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Total and differential leukocyte counts from blood samples obtained from the arterial cannula were made before, immediately after, and at varying times during the recovery phase.

Replacement transfusions were performed on rats weighing approximately 250 grams. In the experimental groups were: (1) 5 rats of the Sherman strain that had been adrenalectomized 1 to 11 days before study, and had been maintained with the subcutaneous administration of 0.5 mg. of desoxycorticosterone acetate (DOCA) daily and 5 per cent glucose in saline for fluid intake; (2) 5 rats of the Sprague-Dawley strain that were hypophysectomized approximately 2 weeks before use*; and (3) 7 rats treated with 5 mg. cortisone acetate (Merck) subcutaneously for 14 days before study. Although extensive experience with normal animals had been gained in previous studies, appropriate sham-operated or injected rats were used as controls. In daily experiments, both an experimental and a control animal were subjected to replacement transfusion with identical donor blood.

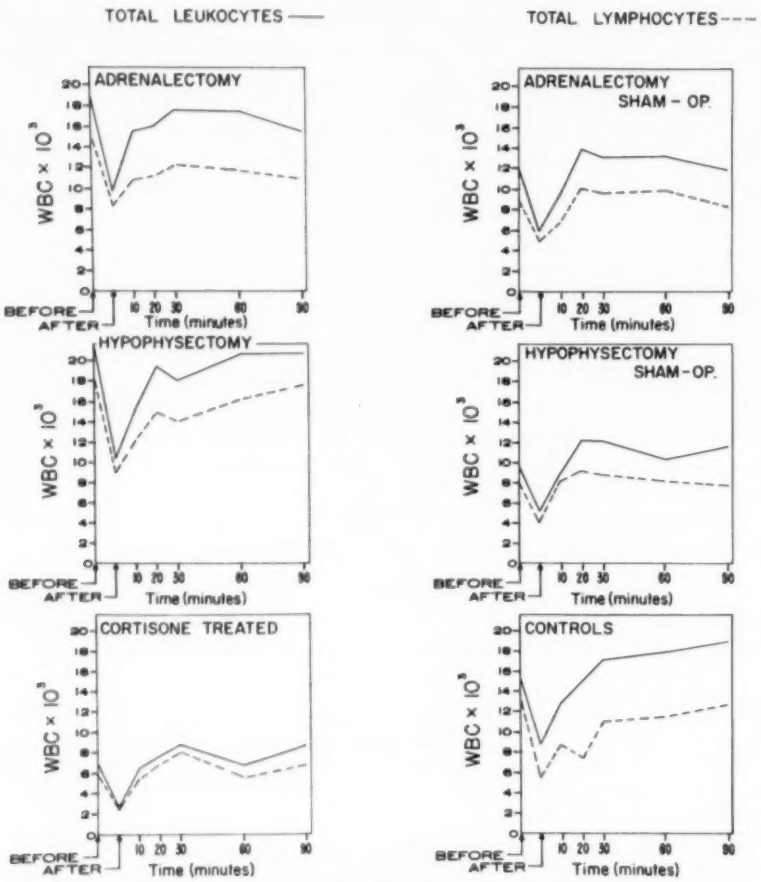

Fig. 1.—Mean changes in leukocytes and lymphocytes during and following leukocyte replacement transfusion.

*Obtained from the Endocrine Laboratories, Madison, Wis.

RESULTS

As anticipated, the peripheral blood leukocytes (predominantly lymphocytes) were increased in adrenalectomized and hypophysectomized animals as a concomitant of their increased lymphatic tissue. Conversely, the cortisone-treated animals had low total leukocyte and lymphocyte levels correlating with the general decrease in lymphoid tissues following adrenal hormone therapy. These differences were marked, with the mean total leukocyte level of the adrenalectomized group 19,100 mm.^{3}; of the hypophysectomized group 21,600 mm.^{3}; and of the cortisone-treated groups only 7,200 mm.^{3}. The marked leukopenia and lymphopenia of the cortisone-treated rats is statistically significant ($P>0.05$), using the ranking test of White,[3] when compared with either the adrenalectomized, hypophysectomized, or the control saline-injected rats.

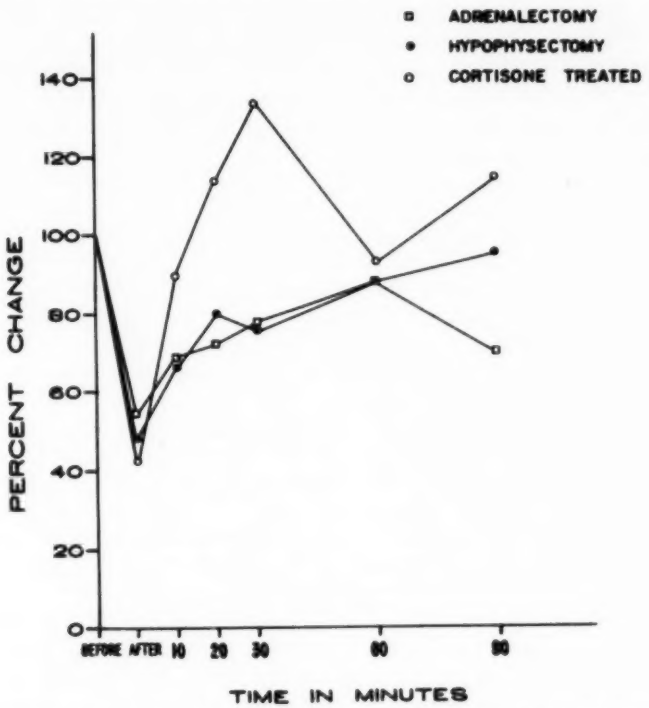

Fig. 2.—Comparison of the percentage change in lymphocytes of adrenalectomized, hypophysectomized, and cortisone-treated rats.

These 3 groups of animals with markedly different lymphatic tissues were subjected to leukocyte replacement transfusion. The results of mean total leukocytes and total lymphocyte changes are shown in Fig. 1. In all groups, considerable mobilization of leukocytes (mostly lymphocytes) occurred during the approximately 5 minutes required to complete the transfusion procedure.

This acute mobilization of cells was manifested by the fact that the calculated 80 per cent blood volume replacement resulted in only 60 per cent or less decrease in leukocytes. Recovery to approximately initial levels occurred in all instances in 20 to 30 minutes, but no significant increases above initial levels were noted. Although the cortisone-treated group had the low total lymphocyte count, the lymphocyte recovery curve, calculated as per cent of initial value, appears even more brisk than in the other groups (Fig. 2). Even the quantitative mobilization during the first 30 minutes (increase of approximately 5,000 lymphocytes per $mm.^3$) is not inconsiderable.

DISCUSSION

Adrenalectomy and hypophysectomy caused the expected increase in peripheral blood lymphocytes in the rat, and cortisone therapy resulted in a marked lymphopenia. When lymphocytes were removed during the course of a total leukocyte replacement transfusion, the cortisone-treated rat exhibited good ability to mobilize lymphocytes back to the initial circulating level. It would seem, then, that cortisone therapy, although altering the total lymphatic tissue, does not significantly change these lymphocytes that we have termed the "ready reserve" for blood entry.

Of interest was the fact that this brisk mobilization of lymphocytes in the cortisone-treated rats did not exceed the initial value. The mobilization, then, ceased rather abruptly at the original lymphopenic level, without any tendency to return to normal or even higher leukocyte levels. We have noted this type of "thermostatic" control of circulating blood leukocyte levels in other studies. The adrenals play a presumably significant role in setting this level for the circulating lymphocytes in the resting animal and, if so, would logically be responsible for controlling the cessation of rapid mobilization in response to our procedure.

In none of our studies to date have we been able to significantly exhaust the leukocyte reserves of the rat, regardless of modification of the animals by operation or drugs. Since the total magnitude of the lymphocyte stores has not been determined by our method, they might be markedly depleted in the cortisone-treated animals that seem to respond so readily. Gowans[4] certainly has demonstrated that chronic removal of lymphocytes from the circulation by thoracic duct drainage does cause eventual lymphopenia.

The significant aspects of these observations, however, is the fact that depletion of circulating lymphocytes by cortisone does not abolish these readily mobilizable cells. The biologic functions of such ready reserves are not clear, but the fact that they seem to remain, even in the animal depleted in lymphoid tissue, suggests that they are of some importance to the rat.

SUMMARY

1. The effects of increased lymphopoiesis (hypophysectomy and adrenalectomy) and of decreased lymphopoiesis (cortisone therapy) on the response of rats to sudden depletion of blood leukocytes by replacement transfusion was studied.

2. Depletion of leukocytes by this technique was followed by a return to essentially base-line levels within 30 minutes as lymphocytes were rapidly mobilized from body stores.

3. Even severely lymphopenic rats (cortisone-treated) exhibited rapid mobilization of lymphocytes, although there was little tendency to exceed the initial blood levels.

We would like to thank Miss Sarah E. Mair for technical assistance in this study.

The actual leukocyte values obtained in the individual experiments are available from the author on request.

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LABORATORY METHODS

PRODUCTION OF MAGNETICALLY CONTROLLED VASCULAR OBSTRUCTION

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IN STUDIES necessitating the occlusion of blood vessels there is a need for a controllable and nontraumatic method of obstruction. Clips or ligatures are frequently used on large vessels, with resulting local trauma. It is possible to obstruct small vessels by the injection of microspheres, which eliminate trauma, but generally obstruct all small vessels.[1] Heat and chemicals have been used to infarct capillary beds, but these are extremely traumatic and may obscure the pathology.

The technique developed here employs a magnetic field applied to the exterior of blood vessels, through which small ferromagnetic particles are passing. The field holds the particles until a sufficient collection completely obstructs the vessel. Early release of the fields eliminates the holding force, and the particles are free to leave the site, while a more prolonged application leads to permanent occlusion of the vessel. Magnetic fields have been used in biologic experiments for the separation of phagocytic cells after they have engulfed ferromagnetic particles,[2] but they have not to our knowledge been used to produce vascular obstructions.

MATERIALS AND METHODS

The method consists of the injection into a suitable blood vessel of a solution of 1 per cent gum acacia in an isotonic saline solution containing a 5 or 10 per cent suspension of magnetic ferric oxide (Fe_3O_4).† The estimated dosage of ferric oxide is approximately 20 mg. per kilogram. Observations suggest that dosages over 50 mg. per kilogram may result in a delayed death due to an excessive accumulation of iron in the lung and reticuloendothelial system. The particles of oxide being smaller than the red corpuscles (1 micron) easily pass through the capillaries and, inappropriate dosage, through the lung without obstruction. After the injection, an electromagnet (described below) is applied over, but not in contact with, the exposed vessel or area to be infarcted. When the vessel is of sufficient size, the accumulation of oxide can be easily visualized beneath the magnet. When the magnetic field is removed prior to the development of a permanent obstruction, the accumulation of

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oxide can be seen to break apart and be carried off by the restored flow of blood. If the magnetic field is promptly reapplied, the oxide may again be accumulated in the field but usually in a smaller quantity.

Two different types of magnets have been employed in this study. Type I is designed to produce a high intensity field in a carefully restricted area for obstruction of a selected vessel. This uses a ¼ inch soft iron rod folded upon itself with the free ends converging, leaving a 4 mm. air gap at the tip. The magnetic field produced by 5,500 turns of No. 28 copper wire wound around the rod is concentrated in the 4 mm. gap between the poles. Power is provided by a 28 v. D.C. 2 amp. power supply. The gap formed by the converging filed ends of the ¼ inch rod is just large enough to admit the blood vessel to be obstructed. The coronary artery was actually dissected free and drawn between the poles by a Teflon tape secured to the magnet. Superficial cerebral vessels may be admitted between the poles by gently pressing the poles again at the brain surface, thereby depressing the tissue adjacent to the vessel.

Magnet Type II is designed to produce a widespread magnetic field for obstruction of all vessels of a tissue area, rather than obstruction of a specified blood vessel. This has been used to infarct vessels in the spinal cord and brain. The original Type II Magnet was a commercial surgical eye magnet, consisting of many turns of copper wire on a straight iron rod. The magnetic field thus produced radiates from the tip of the rod. A new magnet has been prepared with a longer tip for easier application to less accessible areas. An air-cooling coil has also been incorporated into the design so that the field may be applied for a sustained period without overheating.

Fig. 1.—Arterial obstruction in a rabbit's ear, following the discontinuance of the electromagnetic field. The small obstruction between the magnet poles represents a portion of the main block being carried peripherally by the returning blood flow. (Reproduced from a motion-picture frame.)

RESULTS

Preliminary studies with this technique were carried out utilizing a large artery in a rabbit's ear (see Fig. 1). The cerebral cortical veins (Fig. 2) and arteries of 3 large dogs and a rhesus monkey were subjected to temporary and permanent infarctions. In these experiments the oxide was injected into either

Fig. 2.—A cerebral cortical venous obstruction (monkey) between the magnet poles.

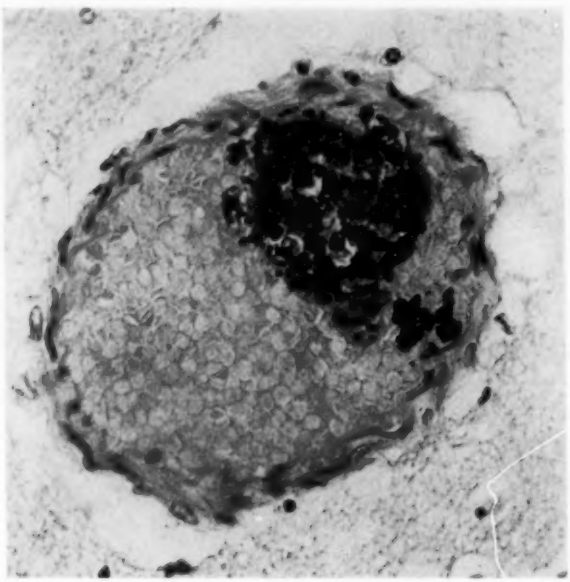

Fig. 3.—A partly obstructed cerebral vein in a localized area of cerebral infarction in a dog. Hematoxylin and eosin stain.

the saphenous vein, pulmonary artery, or carotid artery. The site of injection seems to have no appreciable effect on the time of development or size of the obstruction. When the magnetic field was maintained for 10 minutes or longer the obstruction remained permanent. Limited observations seem to indicate that heparin injections may further prolong this period of reversibility. With Magnet Type II, localized areas of infarction were produced in one cerebral hemisphere of a dog (Figs. 3 and 4).

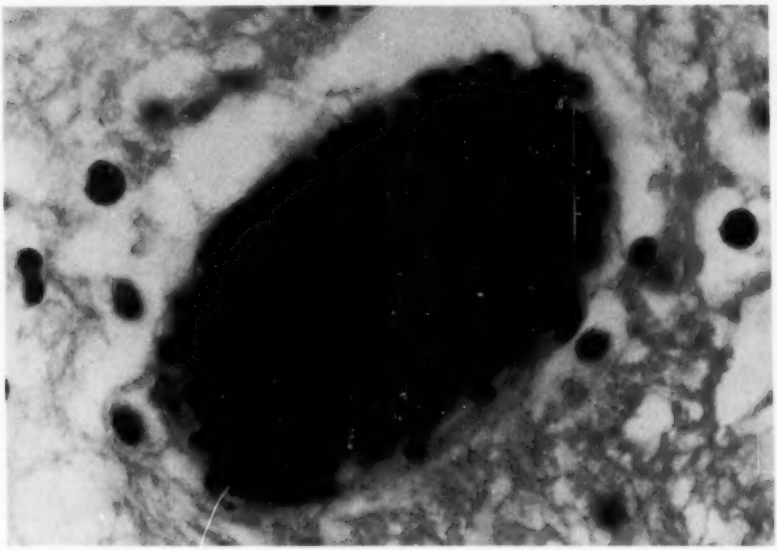

Fig. 4.—A completely obstructed cerebral vein in an area of localized infarction in a dog. Hematoxylin and eosin stain. (Magnification ×640; reduced 3/7.)

Two attempts were made to produce a coronary occlusion in large (30 Kg.) dogs by the injection of 7 ml. of 10 per cent oxide solution into the saphenous vein. The magnetic field was maintained for 15 minutes over the exposed anterior coronary artery. No appreciable accumulation of oxide could be observed, and electrocardiographic recordings showed no change within a 30-minute period of observation. A second injection of 8 ml. was then made and the field maintained for 10 minutes with no demonstrable effect. Electrocardiographic observations over a 1-hour period failed to demonstrate any changes that could be ascribed to the injection. The animal was then sacrificed and histologic studies of the heart also showed no evidence of occlusion. A repetition of this experiment with modification of the magnet shape to permit a better application of the magnetic field was also unsuccessful. It is our opinion, however, that coronary occlusion may be produced by utilizing a stronger magnetic field or in an experimental animal having smaller coronary vessels.

By the application of the magnetic field to the exposed spinal cord (thoracic) of a monkey and a dog it has been possible to produce an area of localized infarction. The occlusions in the superficial vessels of the cord can be easily observed and microscopic studies also demonstrate (Fig. 5) the occlusion of the smaller intraspinal vessels. Further studies on spinal cord infarction are in progress.

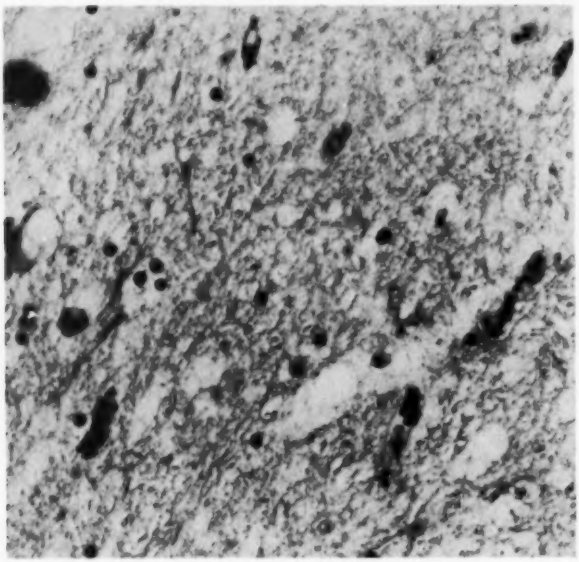

Fig. 5.—Generalized obstruction of all vessels in a localized portion of the midthoracic spinal cord of a dog. Hematoxylin and eosin stain.

Of the 9 animals used in these experiments (1 rabbit, 6 dogs, and 2 monkeys) 3 dogs were subjected to acute experiments and were sacrificed. Two animals expired (1 dog and 1 monkey) following the procedure, the dog within 30 minutes and the monkey after 8 hours. In both cases the dosage of Fe_3O_4 was 50 mg. per kilogram or over. Histologic studies in each case showed a large accumulation of the oxide in the lungs and reticuloendothelial system. The remaining animals showed no ill effects 3 to 4 weeks after the injection.

SUMMARY

A technique for the transient or permanent occlusion of small arteries, veins, and capillary beds is presented. The method is dependent on the accumulation of magnetic ferric oxide when the vessel or tissue area is subjected to an electromagnetic field. The advantage of the technique may reside in the avoidance of trauma to the vessel and adjacent tissue and in the production of controlled infarctions and ischemia. It is suggested that this technique may be of value in studying electrical and chemical changes in small areas of tissue

subjected to periods of transient infarction. It is hoped that the technique can be improved with further development of the electric magnet in regard to the strength and localization of the magnetic field.

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THE ESTIMATION OF ANGIOTENSIN* IN BLOOD

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THE participation of the renin-angiotensin system in experimental renal hypertension has been repeatedly confirmed by direct or indirect evidence.[1-3] However, there still remains some doubt concerning the presence of increased amounts of renin or angiotensin in the blood of animals with renal experimental hypertension and of hypertensive human beings.

Methods used for the determination of renin are either too insensitive or not specific or quantitative enough.[4-7]

Great progress was made by Skeggs and co-workers who developed two methods for the estimation of circulating angiotensin. One of their methods[8] consists in equilibrating the circulating blood of experimentally hypertensive dogs in an artificial kidney with salt solution which is then concentrated and tested for pressor activity. The other method[9] consists in rapidly drawing 250 ml. of blood into alcohol and concentrating and purifying the hypertensin present in the filtrate.

With both of these methods, larger amounts of angiotensin were found in dogs with experimental hypertension than in normal dogs.[8, 9] The same results were obtained in human beings with malignant hypertension.[10] One objection to these methods is that the withdrawal of a large amount of blood (250 ml.) inevitably causes some degree of blood pressure drop which, as shown by Huidobro and Braun-Menéndez,[11] may itself provoke a liberation of renin by the kidney and, hence, the formation of angiotensin in the blood.

In this paper we present an improved method for the direct estimation of circulating angiotensin requiring the withdrawal of only 50 ml. of blood which causes no change in blood pressure in dog or man. Our method has a high rate of recovery and is relatively simple in its manipulations.

METHODS

Principle of the Method.—The blood sample is collected into ethanol and the angiotensin is removed from the filtrate by the resin Dowex 50-X2. On elution with alkali one obtains a pressor-active solution which can be assayed in the rat.

Experimental Procedure.—Fifty milliliters of arterial blood is rapidly withdrawn into 200 ml. of 96 per cent ethanol using a technique closely similar to that described by Skeggs and associates.[9] The mixture is well stirred for 2 to 3 minutes and then filtered

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*Previously identified as angiotonin by Page and collaborators, and as hypertensin by others.

on a Buchner funnel through a bed of Hyflo Supercel. When the cake is becoming dry, but before it cracks, 200 ml. of 80 per cent ethanol is added to the funnel. The washed cake is compressed with an adequate instrument so as to recover most of the withheld liquid. Rinsing the cake with less than 200 ml. of 80 per cent ethanol leads to losses in the recovery of angiotensin. The filtered solution can be kept in the refrigerator overnight, but to avoid losses any precipitate that happens to arise should be included in the next stage.

The alcoholic solution is concentrated at 30 to 35° C. in a rotating evaporator[12] until 270 to 290 ml. of distillate has been collected. The pH of the concentrate is as high as 8 or 9 but this has been found to have no effect on angiotensin. If the filtrate is previously acidified to pH 4 or 5, a heavy precipitate occurs during the concentration and 80 per cent of the angiotensin is lost.

The dark and turbid concentrate is transferred to a separatory funnel, acidified to pH 1 with 5 N hydrochloric acid (approximately 2 ml.), and extracted with 1.5 volumes of ether. The mixture must be thoroughly shaken for 1 minute and allowed to stand for separation. The cycle should be repeated at least twice. The aqueous phase is separated and concentrated to a volume of 50 ml. in the rotary evaporator and adjusted to pH 7.5 to 7.7 using phenol red as external indicator. At this pH there occurs an abundant precipitate that is eliminated by filtration through a thin bed of Hyflo Supercel. The clear filtrate plus a 1 ml. rinse is adjusted to pH 5.6 or 5.7, measured with a glass electrode, and percolated through a column 10 cm. high and 1 cm. wide of Dowex 50-X2, $>$ 120 mesh, equilibrated at pH 5.4.

The resin is prepared according to the method of Hirs and associates.[13] The only difference is the use of the resin retained by the 120 mesh sieve and the molarity of the final equilibrating buffer. We use the 2 normal citrate buffer, pH 5, diluted 8 times. To regenerate the used resin, it is washed on a Buchner funnel with 10 to 20 volumes of 2 N HCl followed by an equal volume of 1 N NaOH and, finally, sufficient quantity of citrate buffer, 0.25 N, pH 5.4, until the filtrate is of the same pH. The resin is stored suspended in the buffer. After repeated use the resin loses its original capacity, and the recovery of added angiotensin may drop somewhat. In this situation it is advisable to stir the resin with 2 N NaOH for 2 to 3 hours on a steam bath. It has been found (Fig. 1) that a column 10 cm. long and 1 cm. wide is able to separate quantitatively the angiotensin present in the extracts prepared, as previously described, from volumes not greater than 50 ml.

In other experiments where the 50 ml. sample was percolated through a 5 by 1 cm. column, the recovery of added angiotensin dropped to 30 to 40 per cent.

The rate of passage should be kept within a range of 0.3 to 0.4 ml. per minute. When all the solution has gone through, the column is rinsed with 10 ml. of water. The elution of the adsorbed angiotensin is carried out with 5 ml. of 0.1 N NaOH followed by 10 ml. of water. This eluate is neutralized to pH 7.5, and the small precipitate that arises is eliminated by filtration. The solution is concentrated to 0.5 to 1.0 ml. and biologically assayed.

The pressor activity of the concentrates was measured in nephrectomized rats anesthetized with Nembutal. The nephrectomy may be performed just before the assay but is more advantageous if done in the previous 24 hours. The registration of the arterial pressure is done in the usual way with a mercury manometer connected to the rat carotid artery by a polyethylene tube. The injections are made in the jugular vein which is cannulated with a short piece of polyethylene tube. The trachea is also cannulated. The sensitivity of the rats is roughly proportional to their weight. One rat unit is the pressor activity of 0.2 ml. of a standard angiotensin preparation (prepared extemporaneously by a four hundred fold dilution of a concentrated solution kept at -20° C.). This amount injected in a rat weighing 200 grams raises its blood pressure 20 to 35 mm. Hg and is equivalent to 0.0135 Goldblatt unit,* and to 0.057 μg of noradrenaline.

*This figure was obtained by comparison with a sample of pure angiotensin I, kindly provided by Dr. L. T. Skeggs, Jr.

We currently use rats weighing 40 to 50 grams; this enables us to detect 0.1 rat unit or less. With these rats it is necessary to inject only small volumes of fluid at a time. We operate within the range 0.01 to 0.1 ml. using a 1 ml. tuberculin syringe driven with a micrometric screw attachment (Fig. 2). The assembly permits the rapid interchange of syringes and is very accurate. One full revolution of the micrometer permits the injection of approximately 0.02 ml. Since all our measurements are relative, we operate in terms of revolutions of the micrometer.

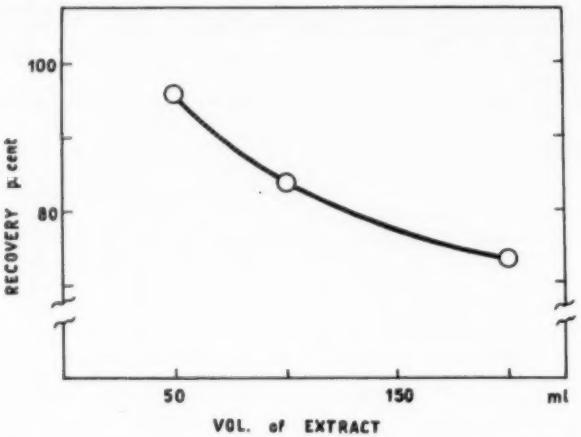

Fig. 1.—Four hundred milliliters of dog blood was processed as described in the experimental procedure up to the stage of passage through the column. The 400 ml. extract obtained was then divided into 4 samples of 50, 50, 100, and 200 ml. One 50 ml. sample was used as a blank and to each of the others 10 units of angiotensin was added. All the samples were then percolated through identical columns of Dowex 50-X2, of 10 cm. per 1 cm. The rest of the procedure was as described. The experimental points represent the recovery of added angiotensin expressed in percentage after deduction of the angiotensin contained in the blank.

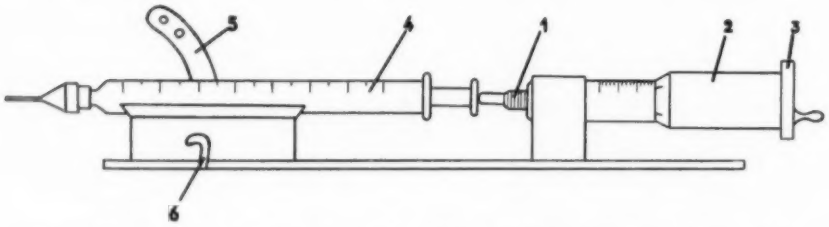

Fig. 2.—Micrometric attachment for the injection of small volumes of fluid to the rat. Key: 1 = micrometric screw of 1 mm. pitch; 2 = barrel graduated in turns and one-fourth turns; 3 = driving head; 4 = 1 ml. tuberculin syringe; 5 = rubber band; 6 = pin to clamp the rubber band and fasten the syringe to the base.

The dead volume that exists in the polyethylene catheter between the blood stream in the jugular vein of the rat and the extreme of the needle of the syringe must be filled with the solution to be assayed before any test injection can be made. Usually this volume does not exceed 0.01 ml.

The routine procedure employed to measure the pressor activity of an unknown solution consists, first, in adjusting the volume injected so as to obtain approximately equal pressor responses in comparison with the standard solution; and, second, in making injections in the following order: 2 or 3 doses of the standard, 2 of the unknown, and the same 2 or 3 doses of the standard. If there is a marked difference between the first and

the last set of standards, the whole series is repeated until the 4 or 6 injections of standard give similar responses. The simple graphical procedure used to determine the activity of the unknown in terms of the standard is explained in Fig. 3.

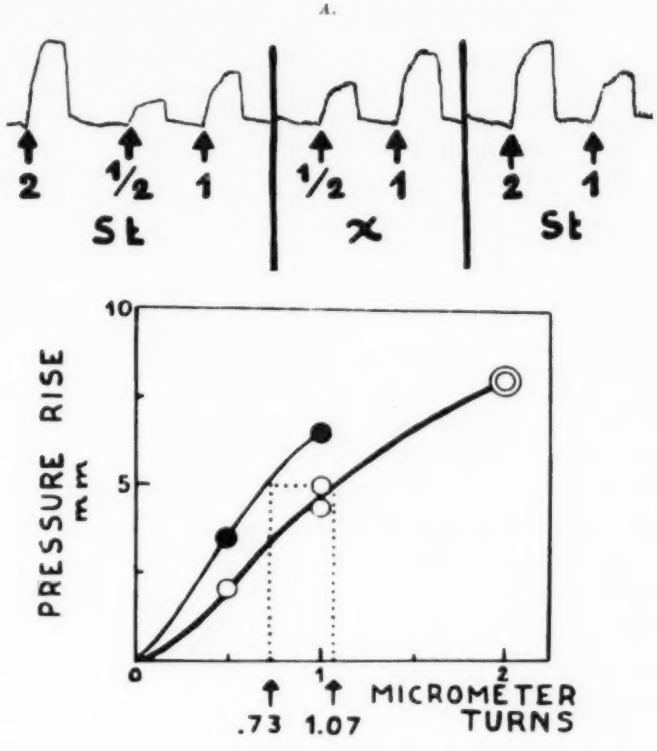

Fig. 3.—Routine procedure to estimate the pressor activity of unknown solutions in terms of an arbitrary standard. *A*, Pressor responses to successive injections of standard angiotensin (5 rat units per milliliter) and unknown solution. The numbers indicate the turns of the micrometric screw corresponding to the injection. *B*, Graphic representation of the pressor responses, measured in millimeters of mercury, as a function of the amount injected. *Open circles*, standard angiotensin points; *black dots*, unknown angiotensin solution points. It is clear that 0.73 of the unknown is equivalent to 1.07 of the standard. Hence the concentration of the unknown is 7.3 rat units per milliliter.

RESULTS

To test the validity of the procedure, 2 accurately made dilutions of the concentrated mother solution of the standard angiotensin were assayed on 11 and 10 animals, respectively, against the usual standard. The results (see Table I) demonstrate that the method has a maximum error of ±14 to 21 per cent on single measurements. The standard deviation in both series does not exceed 11 per cent.

Recovery Experiments.—All the experiments reported were done by adding 10 rat units of angiotensin to 50 ml. of heparinized dog's blood just

prior to its mixing with the ethanol, as explained in the section on Methods. Another 50 ml. of the same blood was processed at the same time and the pressor value obtained was considered a blank of the first sample. Special care was taken to operate with both samples almost simultaneously in the first step to avoid any error due to the observed increase of the blank value that takes place in heparinized blood at room temperature. These data, collected in Table II, show that the method permits a complete recovery of the added angiotensin. The variability observed in single determinations can be explained in terms of the error due to the biologic assay.

TABLE I. REPRODUCIBILITY OF THE ASSAY METHOD

R.U./ML. THEORETICAL	R.U./ML. FOUND (MEAN VALUES)	STANDARD DEVIATION	R.U./ML. EXTREME VALUES FOUND	NO. OF MEASUREMENTS
7.50	7.43	0.64	6.45 8.55	11
3.00	3.24	0.33	2.59 3.63	10

R.U. = rat units.

TABLE II. RECOVERY EXPERIMENTS

NO. OF EXPERIMENT	TOTAL RAT UNITS FOUND	RAT UNITS OF BLANK	RECOVERY (PER CENT)
1	11.4	2.2	92
2	12.0	2.2	98
3	12.7	3.3	94
4	12.5	3.3	92
5	13.6	3.3	103
6	12.4	2.6	98
7	13.5	2.6	109
8	14.2	2.6	116
9	12.3	1.7	106
10	16.2	5.1	111
11	14.4	4.8	96
12	15.9	6.5	96
		Mean recovery	100.9
		Standard deviation	8.1

Specificity of the Method.—Recovery experiments were performed as described before, but other pressor substances were added to the blood. Amounts of adrenaline or noradrenaline equivalent to 25 rat units gave samples devoid of pressor activity. The various alkaline treatments of the procedure must be responsible for their destruction. On the other hand, pepsitensin[14] was completely recoverable, a fact that emphasizes its close similarity to angiotensin. Tyramine and vasopressin* were recoverable only to the extent of 15 per cent of the added amounts. The recovery of serotonin was not attempted since our biologic preparation was constantly insensitive to it. The possibility that tyramine or vasopressin could appear in the final extracts was routinely excluded by testing the resistance of the pressor activity to destruction by trypsin and thioglycollic acid. As is well known, tyramine is unaffected by trypsin while

*Pitressin: Parke, Davis & Company, Detroit, Mich.

vasopressin and angiotensin are completely destroyed. Thioglycollic acid, on the other hand, has no action on tyramine and angiotensin but selectively destroys vasopressin.[15]

The incubating mixture for the trypsin test was as follows:

Extract	0.20 ml.
Phosphate buffer 0.5 M, pH 7.5	0.015 ml.
Merthiolate 4 mg./ml.	0.005 ml.
Crystalline trypsin (Armour) 5 mg./ml.	0.015 ml.

In 10 minutes at 38° C. this proportion of trypsin was sufficient to destroy at least 3 rat units of angiotensin. At the end of the incubation period, the mixture was injected into the rat without further treatment, since it was found that a similar mixture with saline replacing the extract had no effect on the blood pressure of the rat.

For the thioglycollic acid test, the incubating mixture was identical to the previous one but the trypsin solution was replaced by 0.020 ml. of a 0.1 M solution of thioglycollic acid in 0.1 M sodium bicarbonate, final pH 7.5. The incubation time was 30 minutes at room temperature. Under the described conditions, an absolute amount of vasopressin equivalent to 2 rat units of angiotensin is completely inactivated.

DISCUSSION

The problems encountered in the quantitative determination of angiotensin in blood are many. One is that the withdrawal of blood may cause a decrease in blood pressure, thus inducing the secretion of renin by the kidney[11] with the subsequent formation of angiotensin. By reducing the size of the blood samples to 50 ml. this cause of error has been completely eliminated.

The small blood sample, which may eventually be increased to 75 or even 100 ml. according to the size of the animal, creates another problem. The amount of circulating angiotensin which one should expect in hypertensive animals must be very small. Skeggs and associates[9] found from 0.1 to 1.6 Goldblatt units per liter of blood in dogs with acute malignant hypertension and from 0 to 0.05 unit per liter in normal dogs. The fact that the recovery in our method is 100 per cent, while in the method of Skeggs and co-workers[9] it is only about 50 per cent, already increases the sensitivity of our method. The final sample in our method is usually 0.5 ml.; however, it may be reduced to 0.1 ml. and injected totally in a rat weighing 50 grams, thus increasing the sensitivity of the method four- or fivefold in cases where the concentration of angiotensin is expected to be very low. In a rat of this size we can easily detect 0.00054 Goldblatt unit (which would mean for a 50 ml. blood sample 0.01 Goldblatt unit per liter of blood).

The advantage of using a 50 gram rat is that, assuming a concentration of angiotensin of more than 0.03 unit per liter of blood, a fairly good quantitative assay and identification test can be made.

Preliminary experiments have allowed us to determine quantitatively the circulating angiotensin in: (1) nephrectomized animals subjected to a

continuous infusion of angiotensin, (2) normal animals injected with renin, and (3) normal animals subjected to variable periods of deep hypotension.

Experiments are now in progress in which the estimation of angiotensin in blood is made under different physiologic and pathologic conditions.

SUMMARY

A method has been described for the isolation, assay, and identification of angiotensin from the blood. A 50 ml. arterial blood sample is collected into ethanol and the angiotensin removed from the filtrate by the resin Dowex 50-X2. On elution with alkali a solution is obtained which can be further concentrated and assayed for its pressor action by intravenous injection into small rats. The advantages of the method are the small blood sample used, its high recovery rate, the possibility of making identification tests, and its relatively high sensitivity.

We wish to express our appreciation to Miss Elsa Polillo for skillful technical assistance in the biologic assays.

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ADDENDUM

On more extensive use of the method described since our submission of the original manuscript, we have found and solved two experimental difficulties that can lead to low recoveries of angiotensin.

The first problem arose when we used a different batch of Hyflo Supercel which showed a high capacity to adsorb angiotensin. Accordingly, we decided to substitute the second and third filtrations of the method for centrifuging at 12,000 g for 10 minutes in each case. The first filtration was not critical even with the adsorbent batch of Hyflo Supercel.

The second problem arose after the resin had been used repeatedly. Under these circumstances, not even the alkaline treatment in the steam bath could fully restore its original capacity. It is advisable, then, to use fresh resin or alternatively equilibrate the old with citrate buffer 0.2 N, pH 3.1[13] and percolate the sample adjusted to the same pH.

A NEW PRECIPITATION METHOD FOR ESTIMATING SERUM BETA LIPOPROTEINS

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CHARLESTON, S. C.

THE correlation between elevated serum cholesterol, serum beta lipoproteins, and the incidence of thrombotic complication of atherosclerosis has been accepted by most authorities. A simplified method for estimating serum cholesterol and serum beta lipoprotein levels is presented here.

Several years ago it was observed by one of us (E. B.) that a small amount of serum added to a dilute solution of buffered K-agar caused a stable colloidal precipitate. There appeared to be a heavier precipitate when serum of a known hypercholesterolemic patient was added than when the serum of a normocholesterolemic was added. During the course of more than 5,000 measurements, a procedure was developed that makes possible a screening test for hypercholesterolemic and hyperlipemic patients. This procedure correlates well with the Sf 0-16 (beta-1 lipoprotein) component of serum and satisfies the need for a simple, inexpensive (less than 1 cent per test for materials) clinical test to estimate serum cholesterol. It can be used effectively for large scale epidemiologic studies. Somewhat similar, but technically more difficult, methods have been developed by Bernfeld and Nisselbaum,[1] Bernfeld and others,[2, 3] Scanu and associates,[4] and Antoniades and co-workers.[5]

Heretofore, the technical difficulties in separating and estimating serum beta lipoproteins have made this determination impractical for any but highly skilled personnel in elaborately equipped laboratories. Cohn alcohol fractionation, preparative ultracentrifuge differential density flotation with chemical analysis of fractions, analytic ultracentrifugation, and electrophoretic separation are the accepted methods for estimating beta lipoprotein levels. Therefore, this method could fulfill a great need.

MATERIALS AND METHODS

Reagents.—$NaH_2PO_4 \cdot H_2O$ (A.C.S. Reagent grade).
$Na_2HPO_4 \cdot 7\ H_2O$ (A.C.S. Reagent grade).
K-agar, code No. 400 (Baltimore Biological Laboratory, Inc., Baltimore, Md.).

From the Lipid Metabolism Laboratory, Department of Medicine, Medical College of South Carolina, Charleston, S. C.

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**Work done during tenure as a Postgraduate Research Fellow for G. D. Searle & Company.

K-Agar is an extract of carrageenin with a sulfate ash content of 39.1 per cent. Carrageenin,[6] from *Chondrus crispus*, is made up of 1-3′ linked D-galactopyranose units sulfated on carbon 4 with a sulfate ash content of 19 to 23 per cent. The sulfate is bound as a half-ester with the polysaccharide while the remaining portion of the acid is in salt formation with metallic ions, principally sodium, potassium, and calcium. This is significantly different from other agars.

Control Buffer Solution.—Mix equal volumes of 0.0667 M $NaH_2PO_4 \cdot H_2O$ (9.207 Gm. per liter) and 0.0667 M $Na_2HPO_4 \cdot 7\ H_2O$ (17.873 Gm. per liter).

K-Agar Buffer Solution.—Mix equal volumes of 0.0667 M $NaH_2PO_4 \cdot H_2O$ and 0.0667 M $Na_2HPO_4 \cdot 7\ H_2O$. In this solution dissolve enough K-agar to make the concentration of K-agar equal to 0.50 Gm. per liter. This solution should be refrigerated while in storage. Freshly boiled, carbon dioxide-free distilled water should be used to prepare the solutions.

Equipment.—A colorimeter calibrated to read optical density at 540 mμ, cuvettes with an outside diameter of ½ inch, 0.2 and 3.0 ml. pipettes, a pharmaceutical balance for weighing chemicals, and 2 volumetric flasks for making up solutions are the total equipment necessary.

Procedure.—Into the bottom of each of 2 cuvettes, pipette 0.20 ml. of fresh serum. To the first add 3.0 ml. of control solution. To the second add 3.0 ml. of K-agar solution. Mix immediately and read the optical density of each solution at 540 mμ in a colorimeter which has been set with a blank containing control solution without serum. The colloidal solution formed is stable for hours with normal sera, but this is not necessarily true with abnormal sera, so readings should be made within 30 minutes. The optical density reading of the control tube gives the inherent turbidity of the serum which is related to the triglyceride content of the serum. The optical density of the K-agar tube is due to the precipitated K-agar lipoprotein complex plus the inherent turbidity of the serum. Subtracting the optical density of the control tube from the optical density of the K-agar tube gives the optical density difference due to the precipitate and is called hereafter K-agar Δ.

All cholesterol measurements were made using the acid $FeCl_3$ method of Zlatkis and associates[7] with the color reagent modification of Rosenthal and co-workers.[8] Ultracentrifugal lipoprotein separations were made using this laboratory's modification[9] of the method of Bragdon and associates.[10]

RESULTS

During the period of study, several factors were found to influence the nature and quantity of precipitate formed, namely, (1) the quantity of serum, (2) the concentration of K-agar, (3) the pH of the solution, (4) the age of the serum, (5) the temperature to which the stored serum had been subjected, and (6) the character of the metabolic defect, i.e., hypercholesterolemia, hyperlipemia, or diseases which secondarily cause these conditions. Each factor will be discussed separately.

1. There is a simple linear relationship between the quantity of serum used and the optical density of the colloidal solution formed in the range of 0.01 to 0.60 ml. of serum per 3 ml. of reagent.

2. Using a constant quantity of serum, the concentration of the K-agar was varied. The K-agar Δ value was determined for each K-agar concentration and the results are shown in Fig. 1.

At low K-agar concentration, where the ratio of beta lipoprotein to K-agar is relatively high, a high turbidity is found. At high K-agar concentrations, where the ratio of beta lipoprotein to K-agar is relatively low, high turbidity

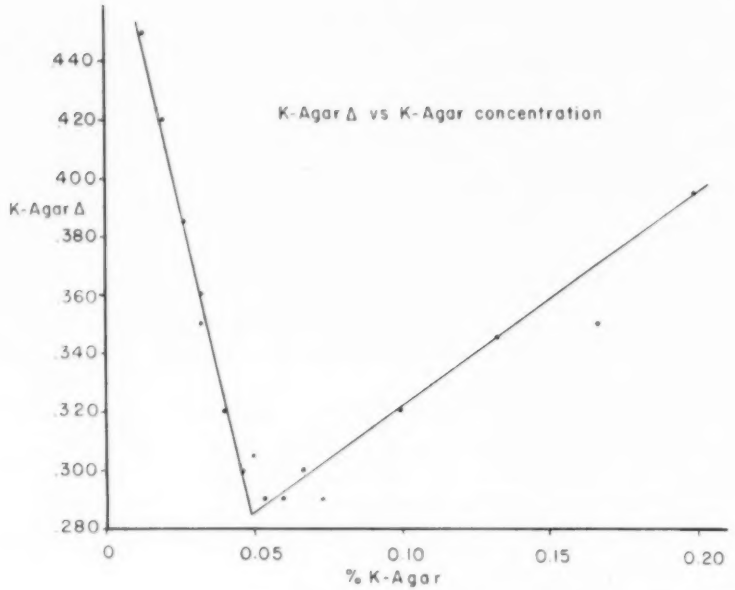

Fig. 1.—K-Agar △ versus K-agar concentration. See text.

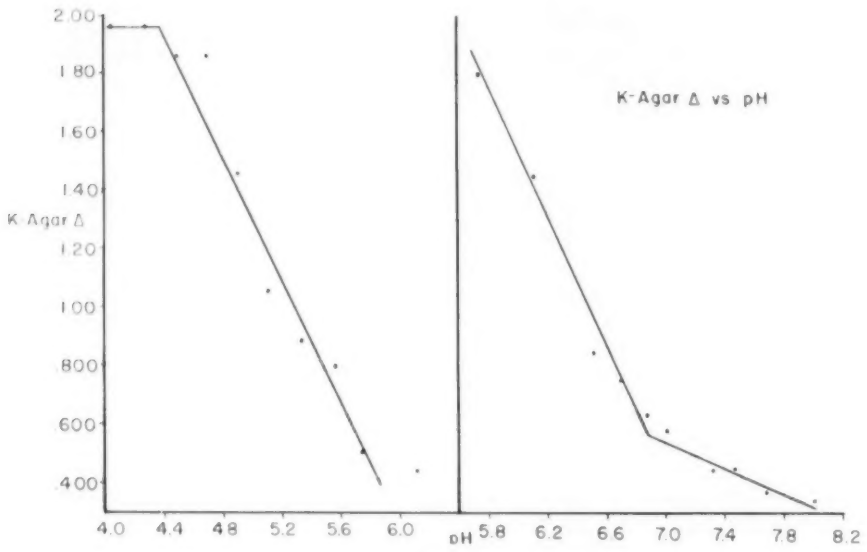

Fig. 2.

values are again found. The curve goes through a minimum at about 0.05 per cent K-agar. This 0.05 per cent concentration was selected for the procedure because effects of technical errors in measurements are minimized.

3. The pH of the precipitation medium is important. Fig. 2 is a typical curve showing the relationship of pH to K-agar Δ values. Many such curves have been determined and each has a characteristic inflection at a pH in the 6.6 to 7.0 range and a second one near pH of 4.6. The K-agar Δ values at the inflection points depend on the concentration of serum beta lipoprotein. The pH chosen for the determination was 6.8 for a number of reasons. At pH values much lower than 6.8 other serum components begin to precipitate. As the pH is raised above 6.8, the amount of precipitate becomes progressively less and the sensitivity of the test is reduced. The advantage of using the 1:1 ratio of salts to make a buffer is obvious in that this ratio gives a maximum stability to the buffering action. When serum is added to the buffer solution there is negligible change in the pH. At pH 6.8 the precipitate is primarily a K-agar beta lipoprotein complex. This precipitate, when separated by centrifugation, is easily soluble in dilute ammonia and shows practically no denaturation. This redissolved precipitate migrates and stains as a beta lipoprotein upon paper strip electrophoresis. If the pH of the K-agar solution is lowered to 4.0, precipitation of all the cholesterol bearing lipoprotein components of serum is complete. Tests are incomplete at this stage as to the exact nature of the precipitate at this lower pH, and as to the serum proteins which remain unprecipitated.

4. Lipoprotein lipase activity and other serum lipases cause changes to continue to occur in serum in vitro; therefore, age and temperature of storage will affect K-agar values. Shown in Fig. 3 are data on two sera, one hyperlipemic and the other hypercholesterolemic. These two terms are here defined as follows: a serum with an elevated Sf 0-16 low density ($> 1.006 < 1.063$ Gm. per milliliter) beta lipoprotein component, called the β_1 fraction for convenience and simplicity, is defined as a hypercholesterolemic serum. Serum with an elevated Sf 17 and above component, very low density beta lipoproteins, called β_2 for convenience and brevity, is defined as a hyperlipemic serum in which the bulk of serum cholesterol is contained in the very low density (< 1.006 Gm. per milliliter) lipoprotein class and has high triglyceride values. This definition is similar to that of Havel and associates.[11] A hyperlipemic serum, even refrigerated, gives about half as much precipitate at the end of 2 days as it does when fresh. A hypercholesterolemic serum remains fairly stable if refrigerated, but deteriorates at room temperature.

Efforts to stabilize serum were made. Gordon and co-workers[12] demonstrated that unesterified fatty acids will bind tightly to albumin. The use of human albumin is shown in Fig. 3. While some stabilization occurred, albumin was generally ineffective. Also tested with negative results were protamine and high ionic strength, which are known to inhibit clearing (Brown and associates[13]).

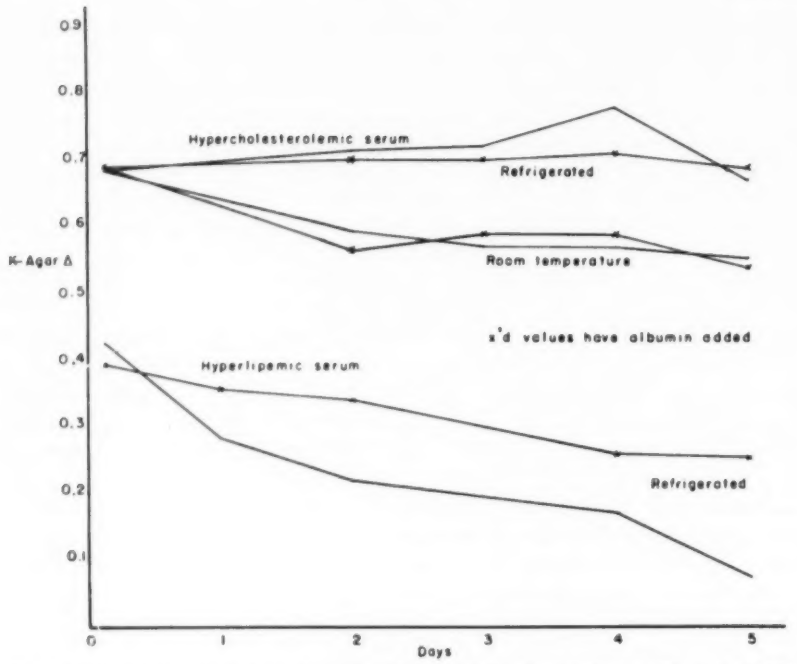

Fig. 3.—K-Agar △ versus time at room and refrigerator temperatures with and without albumin added.

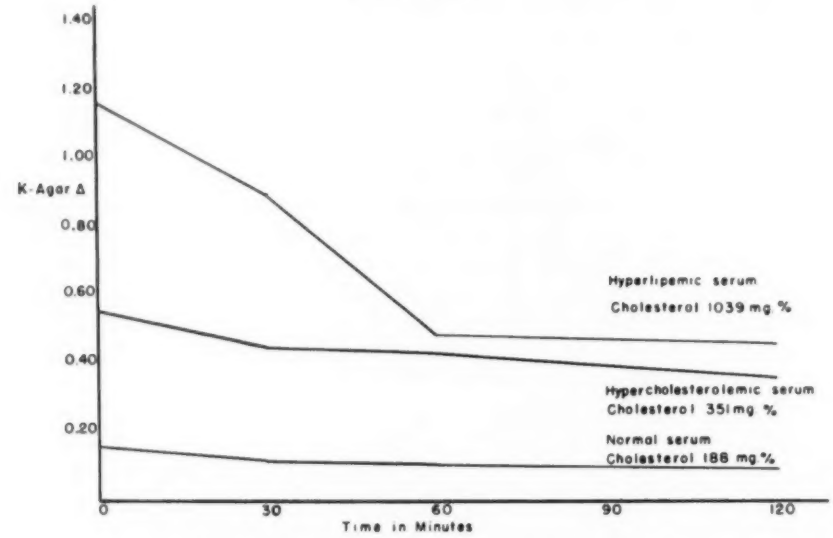

Fig. 4.—K-Agar △ versus time at 37° C. See text.

5. The variation in values attributable to incubating serum at 37° C. before testing is shown in Fig. 4. It is interesting to note again that the hyperlipemic serum is more sensitive to heat than hypercholesterolemic or normal serum. Also, it is significant that serum cholesterol values do not change measurably in this time period. From this discussion, it is apparent that fresh serum must be used for best results using the K-agar test.

Insufficient numbers of hyperlipemic patients and the rapidity of change of the K-agar values with time in such sera prevent the collection of data on the precipitation with K-agar of the nonturbid, very low density, β_2 lipoproteins of the Sf 17-400 class.

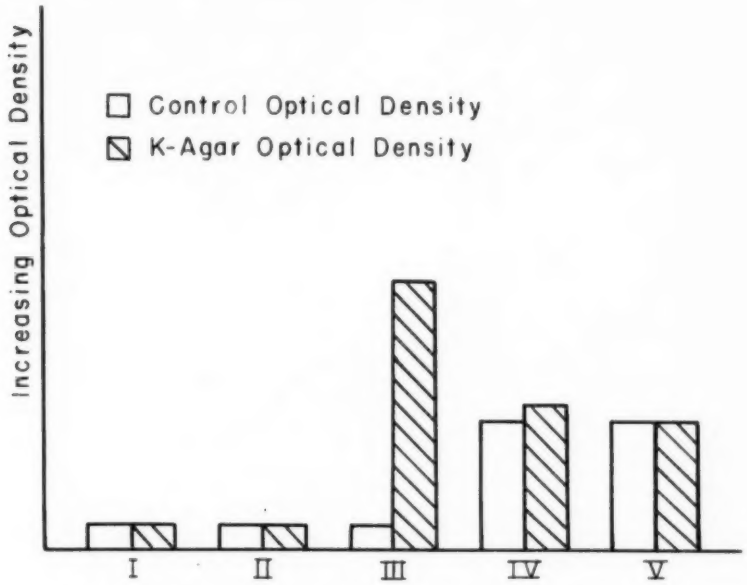

Fig. 5.—Schematic representation of K-agar and control measurement for various lipoproteins. Key: I = lipoprotein-free serum, density >1.21; II = high density, alpha lipoproteins, density <1.21 and >1.063; III = Sf 0-16, beta-1 lipoproteins, density <1.063, >1.006; IV = Sf >17, beta-2 lipoproteins, density <1.006; V = fresh human chyle.

6. In order to characterize the K-agar lipoprotein precipitate, serum was fractionated in a Spinco Model L ultracentrifuge giving (1) a lipoprotein-free component (the infranatant of 1.21 density cut), (2) a high density alpha lipoprotein component (the infranatant of a 1.063 density cut), (3) a Sf 0-16 component, β_1 (the supernatant of a 1.063 density cut), and (4) a Sf 17 and above component, β_2 (the supernatant of a 1.006 density cut). The schematic results of testing each cut using the K-agar test is shown in Fig. 5. Also tested was fresh human chyle.

For each component a control value and a K-agar value is shown. If the difference is taken between these values in each case, K-agar Δ values can be

read. Lipoprotein-free serum and alpha lipoproteins do not cause a K-agar precipitate. The beta-1 component does give a heavy precipitate. The high optical density reading for the beta-2 fraction is due primarily to inherent turbidity as shown by the low K-agar Δ value. Human chyle does not give a detectable K-agar Δ value.

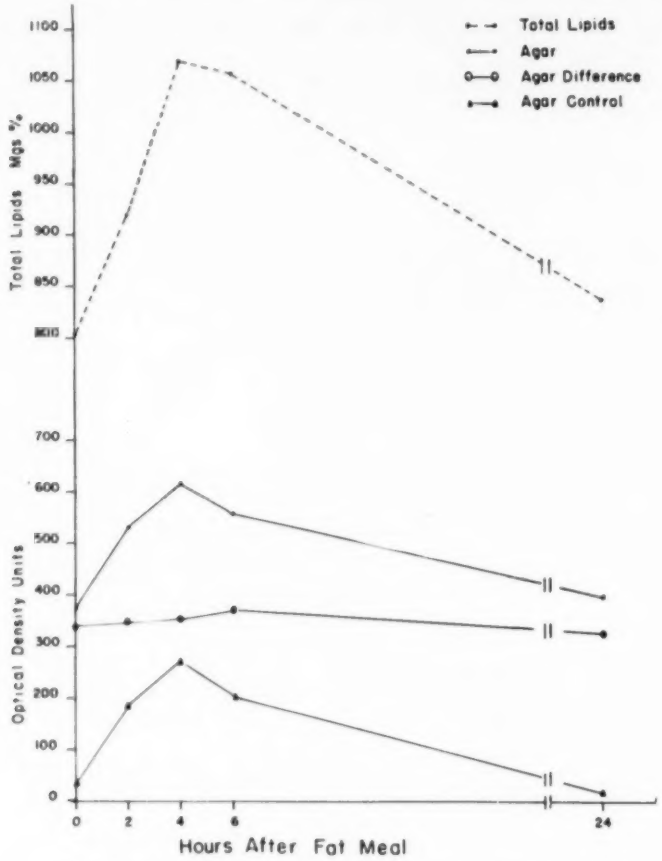

Fig. 6.—Relationship of agar test to alimentary lipemia. See text.

The effect of alimentary lipemia does not destroy the effectiveness of the test. A fat tolerance test was conducted on 24 medical students given orally 1.5 Gm. of fat per kilogram of body weight as a cream milk shake. The composite average of results is shown in Fig. 6.

The broken line shows the serum total lipid values which increase as the fat is absorbed. It is seen that although control and K-agar values increase, K-agar Δ values do not. Although alimentary lipemia does not affect the use

of the K-agar test on normal subjects and hypercholesterolemic patients, it does make more difficult the diagnosis of hyperlipemia. It is recommended, therefore, that the test be made after a high-carbohydrate, low-fat breakfast, so that endogenous mobilization of body fat for energy does not occur. Normal and hypercholesterolemic sera will be clear, and a hyperlipemic serum will be cloudy, ranging from only slightly turbid to very milky.

Fig. 7 is a scattergraph of 163 beta-1 cholesterol values which were plotted against K-agar Δ values. Similar scatter was noted when serum cholesterol or total beta cholesterol values were plotted against the K-agar Δ.

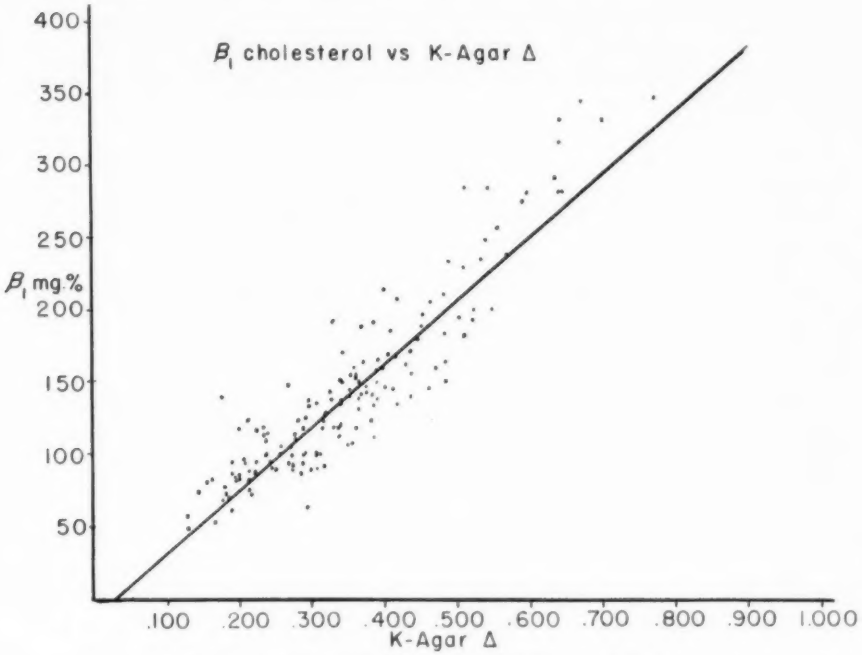

Fig. 7.—Beta-1 cholesterol versus K-agar Δ. See text.

The calculated regression line is shown. In no case was the blood drawn more than 24 hours before the test was made. The tests include sera of normal and some abnormal subjects having either atherosclerosis, biliary cirrhosis, diabetes, or treated or untreated hypercholesterolemia and hyperlipemic patients.

Fig. 8 shows the regression lines for total cholesterol (T.C.), total beta cholesterol (β_1 plus β_2), and beta-1 cholesterol values as plotted against K-agar Δ.

The statistical data are given in Table I.*

*We are indebted to Dr. James A. Gavan, Assistant Professor of Anatomy, for his help in the statistical studies.

TABLE I

REGRESSION EQUATION	REGRESSION COEFFICIENT	STANDARD DEVIATION FROM REGRESSION
T.C. = 493 K-agar Δ + 70	r = 0.934	27.3
$\beta_1 + \beta_2$ = 489 K-agar Δ − 7	r = 0.932	27.2
β_1 = 439 K-agar Δ − 13	r = 0.930	25.0

DISCUSSION

After surveying sera of 1,500 individuals (factory workers, farmers, newborn infants, well-baby clinic patients, student nurses, medical students, hypercholesterolemic patients, and hyperlipemic patients), the upper limit of norms for the following measurements were established: K-agar Δ, 0.350; control, 0.025; serum cholesterol,[7, 8] 240 mg. per cent; total lipids,[14] 900 mg. per cent; alpha cholesterol, 90 mg. per cent; β_1-cholesterol, 140 mg. per cent; β_2-cholesterol, 30 mg. per cent.[9]

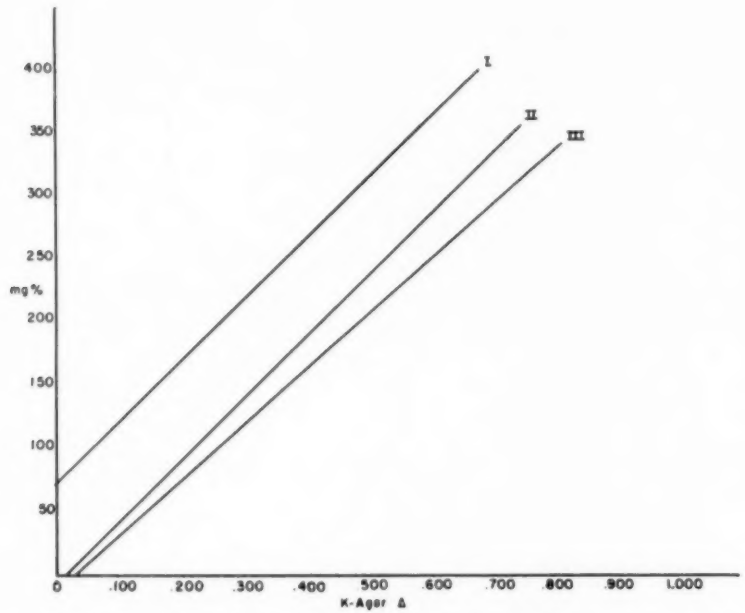

Fig. 8.—Regression lines and cholesterol values versus K-agar △. Key: I = regression line of total serum cholesterol values in milligrams per cent plotted against K-agar △; II = regression line for the total beta cholesterol values; III = regression line for the beta-1 cholesterol values.

The interpretation of results of the K-agar test is made with the aid of the regression lines in Fig. 8. A clear serum is indicative either of a normocholesterolemic serum or of a hypercholesterolemic serum. A clear serum will give a control value in the normal range. Using the K-agar Δ value and the regression lines of Fig. 8, total cholesterol, beta-1 plus beta-2 cholesterol, and

beta-1 cholesterol values may be obtained. From these values, by difference, alpha and beta-2 values may be obtained as alpha plus beta-1 plus beta-2 gives total cholesterol.

An essential hyperlipemic serum will have a cloudy appearance with a characteristically elevated control value. The K-agar Δ value will not correlate well with the regression lines of Fig. 8 and the value is generally lower than expected. The cloudy appearance of the serum after a high carbohydrate, low-fat breakfast is characteristic of a hyperlipemic serum.

In this laboratory a high percentage of sera from patients with lipid metabolic disease is seen. At least 97 per cent of these patients have hypercholesterolemia, most of the rest have idiopathic hyperlipemia. It should be easy for a physician to use this test effectively and with confidence, not only to detect and characterize patients with elevated serum cholesterol, but to follow the results of his treatment.

SUMMARY

A new precipitation test for beta lipoproteins ($<$ 1.063 Gm. per milliliter) has been described using buffered K-agar. Norms have been noted and an interpretation of results has been given. Factors influencing the results are described. Correlation of this test with serum total cholesterol and serum low density beta lipoprotein levels are made. Advantages of such an inexpensive, rapid, simple, and accurate test are described.

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A METHOD FOR PREPARING INTACT ANIMALS FOR CARCASS ANALYSES

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BETHESDA, MD.

CARCASS analysis by sampling from a homogenate of the whole animal has met with little success. The primary difficulty has been the disintegration of the skin.

Chanutin[1] ground the carcass in a meat grinder and took aliquot portions for moisture, nitrogen, fat, and ash analyses. No indication was given as to the agreement between duplicates. However, a statement was made that nitrogen analyses showed a marked variation.

Kochakian and Stettner[2] dried the eviscerated animal to constant weight in an electric oven. The dried residue was dissolved in strong alkali and alcohol. The bones were separated and dissolved in acid. The mixture was made to volume and aliquots of the dissolved carcass were taken for nitrogen and fat analyses. Although this method has proved useful for many purposes, it is cumbersome for large animals. Furthermore, the fat is hydrolyzed by the alkaline hydrolysis making it impossible to do any studies on the original lipids, and the ash cannot be determined accurately.

Another method, similar to the above, involved cutting the carcass into small pieces and digesting the material in strong hydrochloric acid at room temperature.[3] When the bones softened, they were put in large beakers and heated on a water bath for several days. "By appropriate manipulation of the volumes and sampling, a solution was finally obtained representing whole animals and suitable aliquots of this were taken for analysis." This method is rather cumbersome and not readily adaptable to animals having a large amount of body fat.

Other workers[4-6] dried the whole animal to constant weight and extracted the fat from the dried residue. The dried defatted residue was ground, and aliquots were taken for nitrogen and ash analyses. This procedure is time consuming, and no duplicate analyses can be obtained for moisture or fat.

The method to be described obviates most of these difficulties since a homogenate of the entire carcass is prepared. After autoclaving the carcass, a slurry can be prepared in a Waring blendor, and a stable suspension results from passing the slurry through a colloid mill. The homogenate can be used for replicate analyses for the 4 major body components (moisture, protein, fat, and ash) as well as for those specific compounds which are not affected by the autoclaving.

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Received for publication June 26, 1958.

MATERIALS AND METHODS

The special apparatus required for this procedure is an Eppenbach colloid mill.* The Waring blendor containers were of 3 sizes: 250 ml., 1,000 ml., and 1 gallon; the first two fit the regular size blendor; the largest one fits the four-speed model and is needed only for animals weighing over 700 grams.

Preparation of Carcass for Analysis.—The animal was killed by anesthetizing with chloroform, then weighed to the nearest tenth of a gram. The abdomen was opened and the gastrointestinal (GI) tract was removed and freed of its contents which were weighed.† The operation was performed in a frame whose three sides and top were wet towels. This was done to minimize the evaporation of moisture from the exposed tissues. Care was taken to minimize the loss of blood. The GI tract was replaced in the animal and the latter was then weighed. The actual weight of the animal without GI contents was essentially the same as the weight of the anesthetized animal minus the weight of the GI contents; consequently, the latter was used as the "corrected weight" in our calculations of total body composition.

The animal was placed in a wide-mouth Mason jar whose volume in milliliters was at least twice the weight of the animal in grams. The jar was covered and autoclaved for 15 minutes under 15 pounds pressure (120° C.). The animal was then placed in a Waring blendor of appropriate size. Water was added to facilitate homogenization. In our experience, stable homogenates are secured when the volume of water added approximates, for animals weighing up to 500 grams, the corrected body weight or 0.7 times this volume for animals weighing over 500 grams. The exact volume of water does not have to be measured since the weight of the final preparation is used in calculating a factor which corrects for the added water. Approximately one-half the water was gradually added during the 15 to 20 minutes of blending, the remainder was used to rinse out the blendor and colloid mill.

The homogenate was quantitatively transferred to the colloid mill. The blendor was washed with small quantities of water and the washings added to the mill. Before starting the mill the micrometer adjustment device which controls particle size was set for a reading of 40. The water circulating through the homogenizing block was turned on; the colloid mill was started and allowed to operate for about 3 minutes,‡ during which the contents of the mill were stirred to make sure that no particles adhered to the walls of the hopper. The micrometer adjustment device was slowly turned in the direction of "close" (using 10 unit intervals every 5 to 8 minutes) until a reading of "zero" was reached. The mill was allowed to run at zero for another 5 minutes. The colloid mill operation required about 30 to 40 minutes.

The homogenate was then quantitatively transferred to a previously weighed Mason jar. The colloid mill and recycling pipe (if that had been used) were washed several times with small quantities of water and the washings added to the original homogenate. The jar was sealed and the contents mixed by shaking. The homogenate was allowed to cool to room temperature, and the total weight was obtained. Storage at 4° C. followed.

Description of Homogenate.—Five sieves of the following mesh screen sizes, 10, 14, 18, 25, and 35, were stacked in that order with the smallest mesh size at the bottom. Five hundred and thirty-four grams of homogenate equivalent to 315 Gm. of wet carcass were added to the top sieve. The sieves were placed under a faucet and a slow stream of cold water was directed on the homogenate to hasten screening. The material remaining on the screens was dried at room temperature overnight and weighed the following day. All of the homogenate passed through the 10, 14, and 18 mesh screens. Only 0.8 Gm. or

*Manufactured by the Gifford-Wood Company, 420 Lexington Avenue, New York, N. Y.

†The GI contents were removed since these are not really a part of the animal. If it is desirable to analyze the intact animal, that can also be done.

‡When the weight of the animal was less than 100 Gm., a piece of plastic was inserted over the outlet to the recycling pipe and the faucet opened. The homogenate coming out of the faucet was collected in the original Mason jar and poured back into the mill. Recycling in the case of small animals had to be done manually since the volume of homogenate was not enough to fill the recycling pipe.

0.3 per cent of the total remained on the 25 mesh screen and 0.5 Gm. or 0.2 per cent on the 35 mesh screen. The material remaining on these two screens was mainly bone and hair, some of which would have passed through had it not been for the congealed fat in which the bone and hair were enmeshed. The homogenate showed no apparent separation of fat or water even after standing for several days at 4° C.

Analytical Methods.—

Mixing homogenate: Whenever a sample was removed from the homogenate, certain precautions had to be observed. The cold homogenate is in a semicongealed state. In order to hasten heating to room temperature and facilitate mixing, the homogenate was mixed in a Waring blendor. Aliquots were not taken directly from the blendor because consistent, but small, variations between top and bottom aliquots were observed. (Part of the inconsistency in composition might arise from the fact that the slurry practically filled the blendor.) To obviate this difficulty, the homogenate was transferred to the original container which was vigorously shaken just prior to the removal of each aliquot.

A variety of analytic methods may be used on the carcass homogenate. The following are presented primarily to illustrate how the analytic procedures were applied in our laboratory.

Moisture determination: Aliquots equivalent to about 10 Gm. of wet carcass* were placed in aluminum dishes, covered with aluminum foil, and weighed. The aliquots were dried in a vacuum oven at 70° C. to constant weight. This required about 45 hours. The weight of the dried material was obtained and the loss in weight was assumed to be moisture.

Total fat determination: The dried residue from the moisture determination was extracted in a Soxhlet apparatus for 24 hours with petroleum ether (boiling point 35° to 60° C.). The bulk of the solvent was recovered in the upper portion of the apparatus after removing the extraction thimble. The fat in the flask was dried to constant weight in a vacuum oven which was usually accomplished by overnight drying.

Protein determination: One to two grams of homogenate was pipetted (using a large bore pipette) into a 30 ml. Kjeldahl flask and weighed. The nitrogen was determined by a modification of Campbell and Hanna's[7] macromethod. After digestion, the contents of the flask were diluted to a previously calibrated 50 ml. mark and aliquots were taken for distillation.

The small aliquots used for the nitrogen determination were necessitated by the large amounts of fat in the obese rats (those weighing more than 700 grams). If the samples from the obese rats were much larger than the above, excessive foaming occurred which resulted in the loss of part of the sample unless special precautions were taken.

Ash determination: The defatted sample from the fat determination may be ashed or a new aliquot used. In the latter case, 10 to 15 Gm. of homogenate† was weighed in a porcelain crucible (5 cm. diameter). The samples were dried in a vacuum oven overnight. If the sample was from an obese rat, the large amount of fat produced excessive foaming in the muffle furnace with a consequent loss. To avoid this difficulty, the crucibles were heated on a tripod to a dull-red color and the contents ignited with a Bunsen burner. Studies showed that there was no loss of ash as a result of the controlled ignition. The charred residue was then ashed in a muffle furnace at 550° C. until constant weight was obtained (about 24 hours). The sample was cooled in a desiccator to room temperature and weighed.

Estimation of glycogen: Analyses were made to ascertain whether the homogenate could be used for glycogen determination and to secure some indication of the amount of glycogen

*From 20 to 25 Gm. of homogenate had to be used to secure a sample representing 10 Gm. of the wet carcass. This amount could be approximated in 2½ level tablespoonfuls.

†To secure the above weight of sample, 1 to 1½ tablespoonfuls of homogenate were placed in a weighing dish. A piece of aluminum foil was used to cover the crucible during weighing.

in the carcass. Glycogen was extracted from the homogenates of weanling rats by a modification of the method of Good and associates,[8] and analyses for glucose were done thereon by Nelson's modification of the Somogyi method.[9] Using homogenates equivalent to approximately 2 to 3 Gm. of wet carcass, the glycogen content of one rat was 0.16 per cent (individual values: 0.14 and 0.17) and for the other was 0.21 per cent (individual values: 0.19, 0.22, 0.21). These rats were normal animals which had food available until they were sacrificed as described above.

Conversion Factor for Calculations.—Aliquots for the various analyses are corrected for the water added during the preparation of the homogenate by dividing the corrected body weight by the total weight of the homogenate. This factor multiplied by the weight of the aliquot taken gives the weight of wet carcass present in the sample (aliquot taken).

Autoclaving.—To determine whether autoclaving had any influence on the proximate analysis of the carcass, tissue homogenates were prepared with and without autoclaving. The tissues were a lean rat without skin, hair, and GI contents; the adipose tissue from a fat rat; and the muscles of a lean rat. Each of the 3 tissues was homogenized in a Waring blendor and then divided into 6 aliquots. Half of the aliquots were autoclaved and the others were not. Analyses for moisture, fat, and nitrogen showed (Table I) no detectable loss as a result of autoclaving.

TABLE I. EFFECT OF AUTOCLAVING ON MOISTURE, FAT, AND NITROGEN CONTENT

TREATMENT	MOISTURE* (PER CENT)	FAT† (PER CENT)	NITROGEN (MG./GM. HOMOGENATE)‡
Autoclaved	77.41 ± 0.04	98.65 ± 0.03	13.91 ± 0.17
Nonautoclaved	77.29 ± 0.14	98.77 ± 0.02	13.93 ± 0.12

The mean and standard deviations are given. Standard deviation $= \sqrt{\frac{\Sigma d^2}{n-1}}$ where Σd^2 is the sum of the deviations from the mean squared and n is the number of analyses.

*Lean rat without skin, hair, and GI contents.

†Adipose tissue of a fat rat.

‡Muscle tissue of a lean rat. (All values represent averages of 3 determinations each.)

TABLE II. INFLUENCE OF WEIGHT OF SAMPLE ON MOISTURE DETERMINATION

NO. SAMPLES	AV. WT. HOMOGENATE	AV. WT. WET CARCASS* (GM.)	AV. WT. DRY CARCASS (GM.)	MOISTURE	
				AVERAGE	STANDARD DEVIATION†
3	1.0971	0.6355	0.4021	36.73	0.29
3	5.0576	2.9294	1.8512	36.81	0.13
3	10.0375	5.8138	3.6721	36.84	0.12
3	23.8116	13.7917	8.7093	36.85	0.08
2	246.3	142.7	90.50	36.58	0.19

*Weight of rat carcass contained in the homogenate sample.

†See footnote to Table I.

Sample Weight.—Several aliquots of the homogenate of an obese rat were dried in a vacuum oven at 70° C. for approximately 45 hours. The values in Table II indicate that the replicability of the results is not influenced by varying the weight of the homogenate from about 1 to 25 Gm. Within this range, increasing the weight of the sample reduces the standard deviation (from 0.29 to 0.08). There is a suggestion of a slight reduction in moisture content when

very large amounts of homogenate are used. This is probably due to the difficulty in drying such large samples.

Homogeneity.—In order to determine whether the samples were homogeneous, aliquots were taken from the top and bottom of the container. Before each sample was removed from the jar, the contents were thoroughly mixed by hand shaking. After the samples were taken from the top of the jar, approximately one-half of the homogenate was poured into another container. Duplicate aliquots were taken from the homogenate remaining in the original jar and these were called the bottom aliquots. The results (Table III) show very good agreement for the samples from the top and bottom of the container for moisture, fat, protein, and ash. The above results were obtained from the carcass of an obese rat. Similar results were secured on weanling and adult lean rats.

TABLE III. AGREEMENT OF ANALYTIC DATA SECURED ON DUPLICATES TAKEN FROM THE TOP AND BOTTOM OF THE HOMOGENATES OF AN OBESE RAT (CORRECTED BODY WEIGHT = 1,235 GM.)

SAMPLE NO.	WEIGHT HOMOGENATE (GM.)	WT. WET CARCASS (GM.)	WT. DRY CARCASS (GM.)	PORTION OF HOMOGENATE	MOISTURE (PER CENT)
A. Moisture Determination					
1T	12.8656	7.6023	5.5035	Top	27.61
2T	13.4095	7.9237	5.7317	Top	27.66
3B	14.2328	8.4102	6.0795	Bottom	27.71
4B	14.7632	8.7236	6.3080	Bottom	27.68
Average					27.67 ± 0.04*
SAMPLE NO.	WEIGHT HOMOGENATE (GM.)	WT. WET CARCASS (GM.)	MG. N/GM. WET CARCASS	PORTION OF HOMOGENATE	PROTEIN† WET BASIS (PER CENT)
B. Protein Determination					
1PT	1.0646	0.6291	13.83	Top	8.64
2PT	0.9967	0.5890	13.92	Top	8.70
3PB	1.0973	0.6484	13.88	Bottom	8.68
4PB	0.9973	0.5893	13.92	Bottom	8.70
Average					8.68 ± 0.03*
SAMPLE NO.	WT. WET CARCASS (GM.)	WT. EXTRACTED FAT (GM.)		PORTION OF HOMOGENATE	FAT WET BASIS (PER CENT)
C. Total Fat Determination					
1T	7.6023	4.7311		Top	62.33
2T	7.9237	4.9378		Top	62.31
3B	8.4102	5.2377		Bottom	62.28
4B	8.7236	5.4295		Bottom	62.24
Average					62.27 ± 0.01*
SAMPLE NO.	WEIGHT HOMOGENATE (GM.)	WT. WET CARCASS (GM.)	WEIGHT ASH (GM.)	PORTION OF HOMOGENATE	ASH WET BASIS (PER CENT)
D. Total Ash Determination					
5T	11.8170	6.9827	0.0800	Top	1.15
6T	10.2916	6.0813	0.0677	Top	1.11
7B	10.5297	6.2220	0.0707	Bottom	1.14
8B	10.0055	5.9123	0.0699	Bottom	1.18
Average					1.15 ± 0.01*

*For standard deviation see footnote to Table I.
†Protein = 6.25 × nitrogen.

TABLE IV. CARCASS COMPOSITION OF RATS

NO. ANIMALS	AGE (WK.)	SEX	CORRECTED BODY WEIGHT (GM.)	PER CENT OF WET BODY WEIGHT*				$\frac{H_2O}{PROTEIN}$*	TOTAL RECOVERY* (PER CENT)
				MOISTURE	PROTEIN	FAT	ASH		
6	3	M	39.9 (36.2 – 41.6)†	73.09 ± 0.57	16.30 ± 0.10	6.77 ± 0.47	2.80 ± 0.03	4.49 ± 0.06	98.93 ± 0.09
2	4	M	78.7 (75.3 – 82.1)	73.30 ± 0.18	16.69 ± 0.14	6.28 ± 0.14	2.95 ± 0.02	4.40 ± 0.04	99.21 ± 0.11
4	71	F	318.7 (302.7 – 328.2)	57.79 ± 2.05	17.97 ± 0.27	20.93 ± 2.44	3.11 ± 0.27	3.21 ± 0.11	99.80 ± 0.12
2	71	F	702.6 (578.0 – 827.2)	35.89 ± 1.02	10.68 ± 0.99	51.39 ± 1.98	1.93 ± 0.03	3.38 ± 0.22	99.89 ± 0.07
1	44	M	1235.0	27.67	8.68	62.32	1.15	3.19	99.82

*Mean and standard error determined by Mantel's method.[10]
†Range.

These data indicate that if the above procedure is used excellent agreement can be secured in analytic results carried out on all samples of the homogenate.

A further measure of the uniformity of the homogenates was secured from an evaluation of the average standard deviations for the analytic data secured to date. The values listed in Table IV represent the averages for a series of 15 rats. The standard deviations for these separate sets of values have been averaged in Table V. The data in this table show that the average standard deviations for moisture, protein, and fat are between 0.10 and 0.20 while that for ash is less than half of these. Although the data listed in Table VI were compiled for another purpose, it does suggest that on the few animals studied, the standard deviations of the different analytic procedures are greater when carried out on the homogenates from lean rats as compared with those from obese rats, the standard deviations in most cases being at least twice as great.

TABLE V. VARIABILITY OF ANALYTIC RESULTS AS AN INDEX OF THE HOMOGENEITY OF THE CARCASS PREPARATION*

PROCEDURE	RANGE FOR PROCEDURE IN PER CENT	AVERAGE STANDARD DEVIATION†	RANGE OF STANDARD DEVIATIONS
Moisture	27.67 – 74.55	0.10	(0.02 – 0.16)
Protein	8.68 – 18.49	0.18	(0.03 – 0.50)
Fat	5.67 – 62.32	0.10	(0.01 – 0.21)
Ash	1.15 – 3.68	0.04	(0.01 – 0.12)

*Results are based on triplicate or quadruplicate analyses on each of 15 homogenate preparations.

†Average standard deviation $= \sqrt{\frac{n_1 (S.D._1)^2 + \cdots + n_{15} (S.D._{15})^2}{n_1 + \cdots + n_{15}}}$ where $(S.D._1)^2$ through $(S.D._{15})^2$ are the standard deviations squared. n_1 through n_{15} are number of degrees of freedom in each group.[11]

TABLE VI. DAY-TO-DAY VARIATIONS IN RESULTS FOR CARCASS ANALYSIS OF AN OBESE (CORRECTED BODY WEIGHT = 827 GM.) AND A LEAN (CORRECTED BODY WEIGHT = 328 GM.) RAT. ALL ANALYTIC VALUES EXPRESSED ON A WET WEIGHT BASIS

RAT NO.	DAY	MOISTURE (PER CENT)*	PROTEIN† (PER CENT)*	FAT (PER CENT)*	ASH (PER CENT)*	TOTAL RECOVERY (PER CENT)
41,164	0	34.87 ± 0.09	9.69 ± 0.07	53.37 ± 0.06	1.89 ± 0.03	99.82
41,164	5	35.06 ± 0.06	9.70 ± 0.15	53.15 ± 0.06	1.90 ± 0.05	99.81
41,155	0	55.68 ± 0.12	18.10 ± 0.44	23.32 ± 0.42	2.73 ± 0.11	99.83
41,155	5	56.09 ± 0.34	17.91 ± 0.16	23.03 ± 0.26	2.77 ± 0.17	99.80

*Mean and standard deviation are given.

†Protein = 6.25 × nitrogen.

Day-to-Day Variations.—Since the analytic results for many methods show considerable day-to-day variation, an estimate of the latter was secured by carrying out complete carcass analyses on two separate days. The homogenates of two rats were analyzed in triplicate or quadruplicate, with the same analyses being repeated 5 days later. The data in Table VI show that the variation in the values for the results secured on different days is no greater than that for the same day.

Carcass Composition of Rats.—To indicate the application of the method, data are presented for carcass analyses of 15 rats. The results (Table IV) indicate that the sum of the moisture, protein, fat, and ash account for practically all of the animal. On this basis, we have accounted for an average of 99.53 per cent of the rat carcass. It is likely that a part of the material unaccounted for is glycogen, but this is probably no more than 0.2 per cent. Should it be desirable to secure exact values for the glycogen content of the carcass, special precautions would have to be taken to minimize the postmortem loss of glycogen.

The data show that the percentage of body fat in the rat increases with age whereas the water and protein decrease. The ratio of water to protein decreases slightly with age. The percentage of ash in the rat's body decreases with age and shows an even greater decrease as the rat becomes obese.

DISCUSSION

An experienced technician is able to prepare the homogenates for 6 animals and analyze them in duplicate for the 4 major body components (moisture, fat, protein, and ash) by the above technique in a period of 6 days. As far as the homogenates are concerned, from 6 to 8 can be prepared in an 8-hour day. In our experience this is considerably faster than is possible with any other method.

The homogenate is so uniform that the variation between duplicate results is very small. This is especially true for the homogenates of obese and weanling animals.

The sum of the values for the 4 major body components for homogenates of carcasses prepared by this procedure is consistently very close to 100 per cent. The glycogen content can be determined in the homogenate and, under normal circumstances, would account for an average of about 0.1 per cent and probably no more than 0.2 per cent.

The above procedure for preparing carcass homogenates permits duplicate analyses for all methods. This is not possible, especially for moisture and fat, by some of the currently available techniques. The homogenate can be used in any analytic procedure applicable to tissues; the procedures listed in the paper have been given only because they have been found satisfactory for our work. The method of preparing the carcass homogenate should be of value whenever it is desired to determine the carcass content of any compound that is stable to the 15 minutes of autoclaving. Animals showing considerable range in body weight can be prepared by the procedure. We have successfully prepared homogenates of animals ranging in size from weanling rats to rabbits weighing 2 Kg. including guinea pigs and chicks.

SUMMARY

1. A method has been devised for the preparation of homogenates of whole animals. The procedure consists of autoclaving the animal to soften the skin

and other tissues. Preliminary grinding can be accomplished in a Waring blendor. Stable homogenates are prepared by putting the blended mixture through a colloid mill.

2. The homogenate can be prepared in a short time and is so uniform in composition that duplicate analyses for moisture, protein, fat, and ash agree within very small limits. The sums of the values for these carcass components range from 98.9 to 99.9 per cent for different animals.

3. The procedure is applicable to animals with a considerable range in body weight. It has been used in the preparation of homogenates of animals ranging in size from weanling rats to rabbits weighing about 2 kilograms.

We wish to thank Dr. William W. Wright of the Food and Drug Administration for his generosity in making the colloid mill available.

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A RAPID AUTOMATIC SEMIMICRO COLLOID OSMOMETER

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THE measurement of the oncotic (colloid osmotic) pressure of solutions of substances with high molecular weights has long been of interest to workers in biologic and physicochemical fields. The earlier methods of determination have been reviewed by Petit[1] and Briggs.[2] These methods, as a rule, have been very time consuming and have been inapplicable to the rapid measurement of large numbers of samples.

Meehan and associates[3] constructed a colloid osmometer utilizing an unbonded strain gage transducer. The osmometer was found to be impractical, however, due to technical difficulties[4] which included: (1) excessive time required to reach equilibrium, and (2) large volume displacement of the system due to movement of membrane. Further work on it was discontinued. Hansen[5] has developed a colloid osmometer which registers, by means of an electric condenser manometer, the balancing pressure which prevents solvent flow across a membrane into the colloidal solution. However, his device requires repeated balancing of the oncotic pressure during each determination with an externally applied hydrostatic pressure from a fluid filled manometer. Rowe[6, 7] has constructed an osmometer similar in principle to the one developed by Hansen. It also requires balancing with a variable external hydrostatic counter pressure during the course of each determination. The point of no fluid transfer or balance of oncotic pressure with external hydrostatic counter pressure is determined visually from serial galvanometer readings. The corresponding osmotic pressure is then read on the balancing hydrostatic manometer. The time for each determination is about 40 minutes.[8]

We have developed a fully automatic semimicro colloid osmometer which permits determinations in as little as 5 minutes. This instrument is capable of recording colloid osmotic pressure of small samples (0.5 to as low as 0.1 cm.^{3}). The pressure (negative with respect to atmospheric pressure) produced in a sealed solvent-filled chamber by the passage of solvent across a semipermeable membrane into the colloidal solution is recorded by a strain gage. Significant dilution of the colloidal solution by the passage of solvent into it does not occur since the coefficient of volume change with pressure change (dV/dP) for the sealed solvent-filled transducer chamber is negligible.

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MATERIALS AND METHODS

The osmometer utilizes a Statham P23D pressure transducer* as the pressure sensing device. The pressure dome of this transducer is a removable plastic fitting that has two standard Luer-Lok male adapters; one on the top center, the other on the side. The orientation of parts of the osmometer is shown in Fig. 1. Figs. 2 and 3 show the assembled instrument.

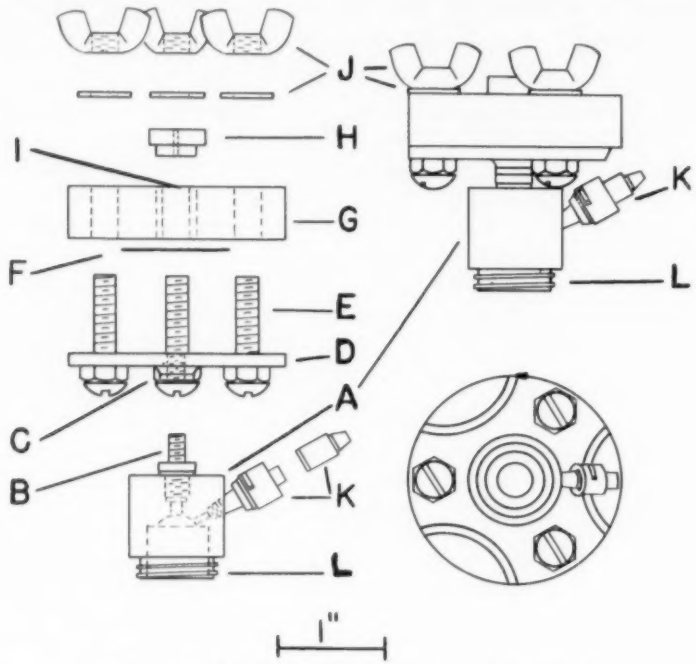

Fig. 1.—Exploded and assembled views of osmometer.

Construction.—The Luer-Lok collar is removed from the top male adapter and the male connection is threaded with an 8-32 S.A.E. thread (Fig. 1, B). A ⅛ in. thick brass plate, 2 in. in diameter (D), is soldered to a ⅛ in. thick brass spacer ¼ in. in diameter (C). This is then tapped in its center through the spacer and the plate to receive the threaded male adapter (B) from the pressure dome. Three spaces are cut out of the brass plate (D) to provide clearance for the side arm Luer-Lok male adapter (K) on the pressure dome. In addition, 3 tapped screw holes, 120 degrees apart, are placed ¾ in. from the center of the brass plate (D) to receive three 12-24 brass screws (E). The brass plate (D) which is to act as a platform on which a semipermeable membrane (F) is to be placed is then screwed tightly to the top threaded male adapter (B) of the pressure dome (A).

Care is taken that the side arm Luer-Lok male adapter of the pressure dome is underlying 1 of the 3 clearance spaces that have been cut out of the brass plate. This provides sufficient working room when the side arm is to be used. The spacer that has been soldered to the brass plate provides sufficient clearance for the side arm as the brass plate is being screwed to the top threaded adapter of the pressure dome. When the plate and pressure dome have been firmly connected, the surfaces of the brass plate (D) and any of the threaded top male adapter (B) that is protruding through the plate are ground and polished on stones

*Statham Instruments, Inc., 12401 West Olympic Boulevard, Los Angeles, Calif.

and emery cloth of progressive fineness until a highly smooth flat surface is obtained. This surface is to receive the membrane (F).

A ¼ in. thick Lucite plate, 2 in. in diameter (G), having a center hole (I) ⅜ in. in diameter is prepared with three ¼ in. diameter clearance holes 120 degrees apart, ⅝ in. from the center of the plate. These holes receive the three 12-24 brass screws (E) from the brass plate.

Fig. 2.

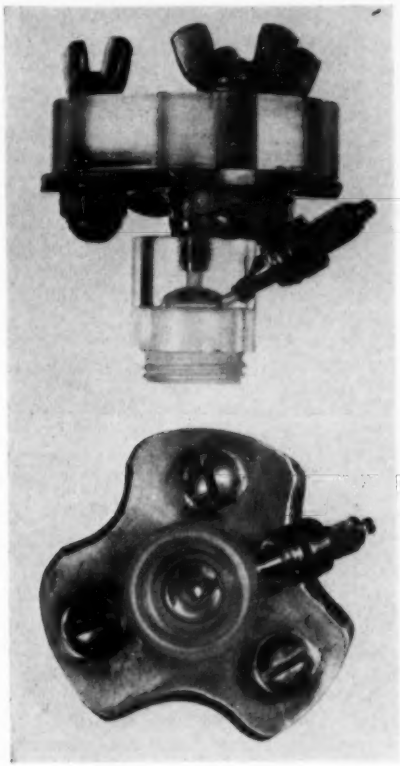

Fig. 3.

Figs. 2 and 3.—Full size photograph of osmometer.

The Lucite plate is then highly polished in the same manner as the brass plate. It serves to hold the membrane on the brass plate and provides a chamber (the ⅜ in. center hole) into which the colloidal solution is placed (I). By varying the diameter of the center hole in the Lucite, chambers of varying capacity may be prepared. The Lucite plate is held in place by wing nuts (J) on each of the 3 screws (E) from the brass plate.

A ¼ in. thick Lucite cover (H), ½ in. in diameter with a center hole 0.5 mm. in diameter, is made for the solution chamber (I). The cover prevents evaporation of colloidal solution within the chamber. The center hole in the cover permits equilibration of pressure within the chamber with atmospheric pressure.

The P23D pressure transducer is powered by a 12-volt storage battery. The electrical output from the transducer is fed into a Statham control box with a heliopot introduced

into the zero adjustment circuit. The electrical output from the control box is then connected with a Brown potentiometer recorder with an input sensitivity of 5 mv. full scale.*

The electrical recording system is calibrated directly against pressure by means of a water manometer that is attached to the side arm (K) of the fluid-filled transducer pressure dome. This pressure calibration is done only once since an electrical calibrator on the control box can be used for further calibration once its pressure equivalent in terms of millimeters of water has been determined (Fig. 4).

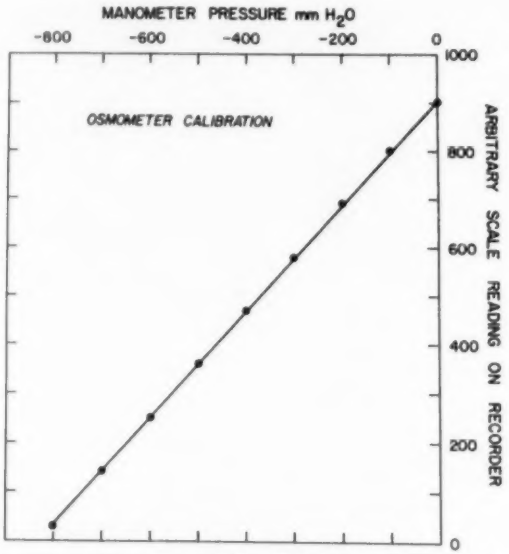

Fig. 4.—Osmometer calibration.

The membranes that we have found satisfactory are commercially prepared.† The 20 mm. diameter size, ultrafine, dense, and very dense, are suitable for the determination of the oncotic pressure of human serum albumin. The membranes can be left in the osmometer and used for many determinations. We have used them for well over 100 runs each and found no leakage of protein across the membrane into the sealed solvent chamber when tested with 10 per cent trichloroacetic or 6 per cent sulfosalicylic acid. In fact, the more often the membrane has been used the more suitable for rapid determinations it becomes since its rigidity and adjustment to the osmometer are improved with use.

Methods.—1. The plastic pressure dome (Fig. 1, A) with attached brass plate (D) is immersed in a solvent bath and filled to the exclusion of all air. Care must be taken that no bubbles persist in the dome. This is easily accomplished by flushing any bubbles with a syringe attached to K.

2. A cap is placed firmly on the Luer-Lok male side arm of the pressure dome (K).

3. The transducer element itself is immersed in the solvent. This can be done with the P23D because the back of the transducer and electrical elements are protected by a plastic jacket extending over the power cord. After all bubbles have been flushed off, the transducer element is placed into position and screwed firmly to the pressure dome at L. This is all done while still immersed in solvent.

*Model No. Y153x(62)P12-x-(16) was used in these experiments with all 12 leads recording. Calculations were based on the plus marks which are identifiable on the original records.

†Carl Schleicher & Schuell Company, Keene, N. H.

4. The assembled transducer element and attached pressure dome with sealed side arm is then removed from the solvent.

5. The membrane (F) is placed on the brass plate (D) over the center hole (I) making sure there are no trapped bubbles underneath it. The membrane is kept moist at all times.

6. The Lucite plate (G) is placed in position on the brass plate (D) and firmly attached by means of wing nuts (J) placed on the screws (E) protruding from the brass plate.

7. The assembled osmometer is now placed in a suitable protecting bag (polyethylene serves well) and immersed in a constant temperature bath.

8. After a zero base line has been obtained, the Lucite chamber (I) is cleared of all fluid (best accomplished by filter paper strips) and the sample is placed in the chamber. Samples of 0.5 to as low as 0.1 cm.^{3} can be used with the size chamber described.

9. A drop or two of sample is placed on the underside of the small cover of the Lucite chamber (H), taking care not to cover the tiny hole. This is done to prevent evaporative loss of solvent from the solution in the osmometer chamber.

10. The cover (H) is placed on the sample chamber of the osmometer.

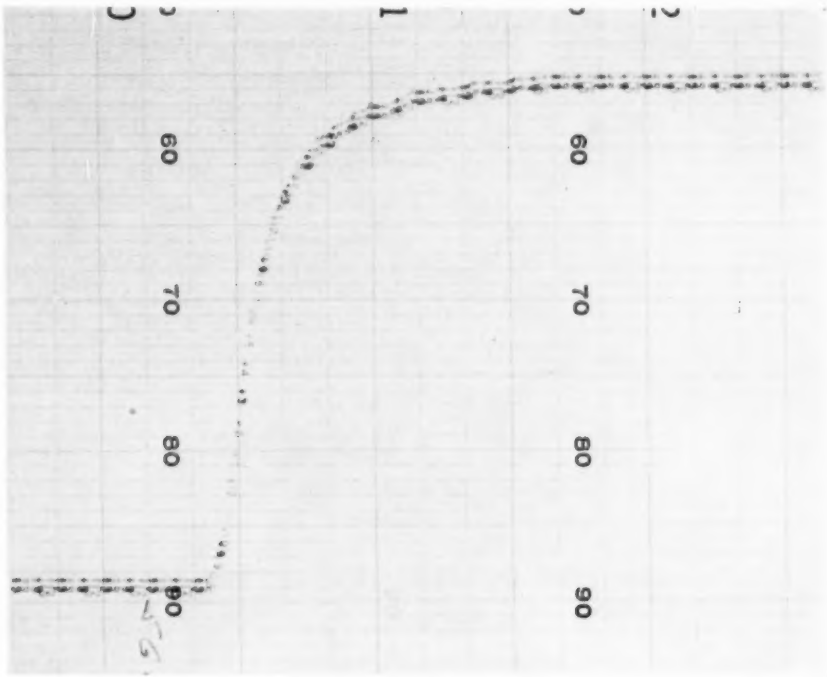

Fig. 5.—Sample record to approach to equilibrium. Record reads from left to right.

11. The oncotic pressure of the sample is represented by a plateau in the record (Fig. 5). The first determination from the zero base line usually takes about 15 to 20 minutes.

12. If serial determinations of solutions with similar solvents are to be run, all that is necessary is to remove the solution from the chamber, flush the chamber a few times with solvent, blot it dry with filter paper, and add the new solution. Serial determinations may be accomplished within a few minutes each following the first determination since the membrane has by then become set and adjusted to the osmometer.

RESULTS

The standard error of the method based on 8 determinations on human serum was ± 3.5 mm. H_2O from an average value of 345.1 mm.

The approach to equilibrium can be very easily followed since a continuous pressure recording is made (Fig. 5). When the logarithm of the pressure differential from equilibrium ($P\text{-}P_{EQ}$) is plotted against time (t), a straight line is obtained. This is to be expected since the rate of approach to equilibrium (dp/dt) is proportional to the pressure differential from equilibrium $\left[\frac{dp}{dt} = k(P\text{-}P_{EQ})\right]$ (Fig. 6). The same oncotic pressure is obtained when it is approached from pressures either above or below the equilibrium pressure.

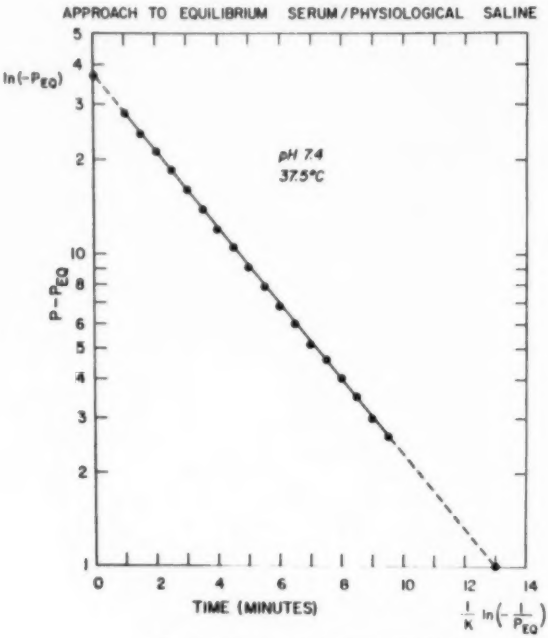

Fig. 6.—L_m approach to equilibrium.

DISCUSSION

This osmometer is particularly suited to serial determinations of oncotic pressure. Once a zero base line has been established with solvent on both sides of the membrane, the first determination will bring the osmometer into a state whereby further determinations can be done rapidly without bringing the instrument back to the zero base line. By starting at a pressure near the oncotic pressure of the sample, much time may be saved during each run.

The amount of solvent transfer across the membrane into the sample during the approach to equilibrium is negligible, even when the determination is started from the zero base line. A demonstration of this negligible fluid transfer is that serial determinations of the same sample of colloidal solution give the same oncotic pressure reading when the run is started from the zero base line as when it is started at a pressure very close or equal to the equilibrium pressure. In addition, solution aspirated from the sample chamber, after equilibrium has been reached, gives the same oncotic pressure when it is redetermined starting from the zero base line.

The rate of pressure approach to equilibrium (dp/dt) has been shown to be a function of a rate constant (k) and the pressure differential from equilibrium ($P\text{-}P_{EQ}$). $dp/dt = k(P\text{-}P_{EQ})$.

Also, dp/dt can be expressed as a function of two other quantities which, in turn, are functions of several other parameters.

A. The rate of transfer of solvent through the membrane $(\delta V/\delta t)_p$:

(1) varies directly with the membrane diffusion constant for the solvent involved;
(2) varies directly with the membrane area;
(3) varies inversely with the membrane thickness.

B. The volume elasticity of the sealed solvent chamber $(\delta P/\delta V)_t$:

(1) varies directly with the rigidity of the membrane;
(2) varies directly with the rigidity of the rest of the sealed plastic solvent chamber; however, this factor is negligible compared to the rigidity of the membrane.

$$dp/dt = (\delta V/\delta t)_p \, (\delta P/\delta V)_t = k(P\text{-}P_{EQ})$$

Therefore, k depends upon the membrane factors in the following manner:

$$k \propto \left\{ \begin{array}{l} \text{membrane diffusion constant} \\ \text{membrane area} \\ \dfrac{\text{membrane rigidity}}{\text{membrane thickness}} \end{array} \right.$$

One might suspect that the largest value for k would be obtained by using a very thin membrane with a large surface area exposed to solvent. However, membrane rigidity plays a very important role and the most rigid membrane would be a thick one with a small surface area exposed to solvent.

Meehan and associates[3] attempted to solve the problem of membrane rigidity by using a perforated metal disk to support a thin cellophane membrane with a relatively large surface area. However, even with the disk, the system was not rigid enough to prevent excessive fluid transfer from solvent to sample chamber.

We have found that an unsupported thicker membrane with a small area exposed to solvent provides a rapid and satisfactory arrangement.

When a new membrane is used, there is usually an initial lag in the pressure curve due to the adjustment and settling into position of the membrane

as negative pressure begins to develop in the sealed solvent chamber of the transducer.

SUMMARY

A colloid osmometer* has been described with the following advantageous characteristics:

1. It is entirely automatic, i.e., no balancing of oncotic pressure with an externally applied pressure is necessary.

2. A complete pressure-time tracing of the approach to equilibrium is obtained so that the course may be followed during the actual run.

3. Serial determinations may be easily and very rapidly performed.

4. The instrument is capable of semimicrodeterminations on volumes from 0.5 to 0.1 cm.^{3} By making a smaller solution chamber, the volume of solution needed can be made correspondingly less.

We are indebted to Dr. Milton Landowne for keen and perceptive comments regarding the osmometer and to Mr. Raymond Flath for his aid with the electric recording system.

We would also like to thank Dr. Nathan W. Shock and Dr. Bernard L. Strehler for reviewing this manuscript.

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*Patent pending.

QUANTITATIVE BACTERIOLOGIC STUDY OF TISSUES, FLUIDS, AND EXUDATES

Some Simplified Methods

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Edgewood, Md.

IT IS good to know when there is sugar in the urine. It is better to know *how much*, and it is best that this knowledge be attained while the information is still of immediate clinical value. Except in complex studies, it usually suffices to report the glucosuria on an approximate scale of "trace" to "4+," rather than in milligrams per cent.

In comparison with this simple example from clinical chemistry, bacteriology lags behind. Clinical bacteriology is still largely a qualitative process, and the time customarily required for making a qualitative determination is more often measured by calendar than by stop watch. Pseudoquantitative descriptions, such as "profuse growth" or "predominant organism" give little real information and sometimes can actually be misleading. Quantitative bacteriologic determinations can be had, however, at the expense of more time and a considerable laboratory effort. There appears to be no commonly used bacteriologic counterpart of the "plus" reading on urine sugar.

For many types of organisms of clinical and laboratory importance, it is possible, by the incorporation of a few simple steps into the laboratory regimen, to obtain quantitative bacteriologic readings as a matter of routine. These readings will be estimations or approximations, but they certainly can be as useful and as dependable as are "plus" readings for sugar in the urine. Also, as long as a presumptive or generic or familial identification of the organism is sufficiently useful, these readings can often be obtained within 12 to 24 hours after the specimen is taken.

My experience with the methods to be described is limited to the study of infectious processes in laboratory animals, but there does not appear to be any reason why these methods should not be of clinical value.

MATERIALS AND METHODS

Materials—Platinum loop, 0.01 ml. (Fisher Scientific Company, No. 13-103). Standard cotton-tipped swabs (Q-tips, Inc., Long Island City, N. Y.). Pipettes and other standard laboratory glassware.

From the Biophysics Division, Chemical Warfare Laboratories, Army Chemical Center, Maryland, and the Division of Surgery, Walter Reed Army Institute of Research, Washington, D. C.

The data presented and the opinions expressed herein are the responsibility of the author as an individual, and are not to be construed as reflecting the official position of the United States Army or any element thereof.

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Preparation of Specimen.—The specimen is used undiluted, or prepared in a dilution which will place the estimated bacterial concentration within the sensitivity range of the smear and plate methods. Liquid specimens (blood or urine) are measured by pipettes; solid specimens are triturated after measurement by volume or weight. Rectal swabs or swabs of wounds can be considered as representing a sample of 0.1 Gm. or 0.1 ml. Most often, we wring out such swabs in a tube containing 0.9 ml. of trypticase soy broth, making a 10^{-1} dilution.

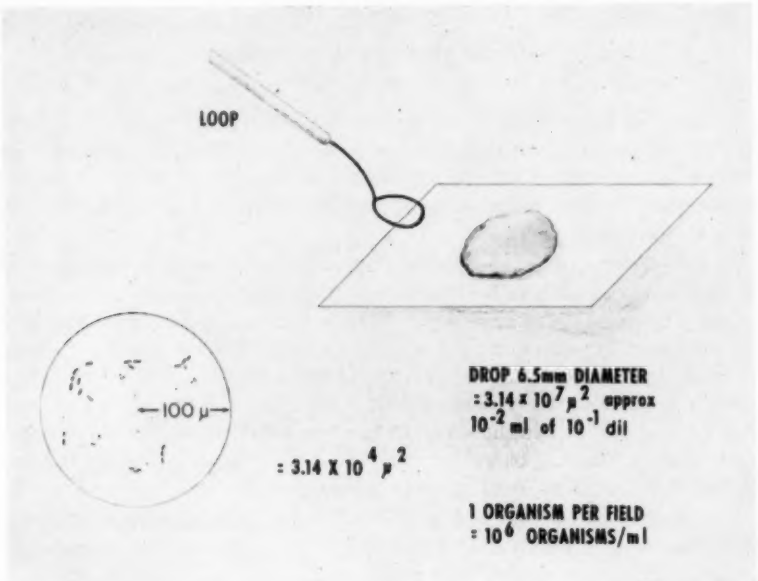

Fig. 1.—Quantitative smear technique, using drop of standard diameter placed on slide by standard-volume loop.

A. *B.* *C.*

Fig. 2.—Successive steps in serial dilution streaking of single plate for surface plate estimation.

Smears.—One standard 0.01 ml. loopful of prepared material is placed carefully on a glass slide, dried in air without heat, and fixed in 95 per cent ethanol. When the diameter of the microscopic field is known (and it can be determined with sufficient accuracy by use of a bacteriologic or hematologic counting chamber) it is easy to calculate a scale for the

number of organisms per average or typical field in relation to the concentration of organisms in the specimen. We use a microscope with an oil immersion field approximately 100 μ in radius. The loop delivers a drop approximately 3.25 mm. in radius. For all practical purposes, with a specimen diluted to 10^{-1}, one organism per field represents 10^6 organisms per milliliter in the original specimen (Fig. 1).

Plates.—One standard 0.01 ml. loopful of prepared material is spread evenly over one-half of an agar plate 100 mm. in diameter (Fig. 2, *A*). The loop is flamed, and a diagonal streak approximately 5 cm. long is made with the flat of the loop through this inoculated half. The inoculum so picked up is spread evenly over one of the lower quadrants of the plate (Fig. 2, *B*). The loop is again flamed; the inoculated quadrant is passed through with a streak approximately 2.5 cm. long, and this inoculum is spread in the remaining quadrant (Fig. 2, *C*). After incubation, plates are read quantitatively by a scale such as that shown in Fig. 3.

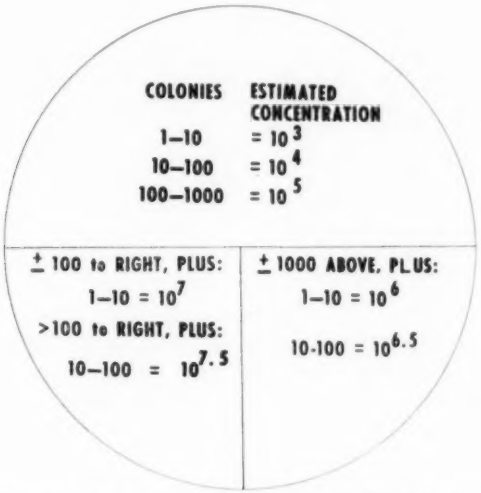

Fig. 3.—Scale for reading serial dilution streaked plates.

RESULTS

By the use of the quantitative smear technique it is possible, within one hour from the time the specimen is taken, to render a report which includes useful quantitative details. For example:

> A direct smear examination of the specimen indicates the presence of approximately 10^7 gram-positive cocci, 10^8 gram-positive rods, and 10^8 gram-negative rods per milliliter. Most of the cocci are morphologically suggestive of fecal streptococci. The gram-positive rods are about equally divided between large blunt forms in which no spores are seen, and smaller rods which show occasional subterminal oval spores with swelling of the rod. The gram-negative rods are all minute pleomorphic forms. Several degenerated muscle fibers are seen. Culture data will be reported.

Quantitative estimations on smears are reported only to the nearest $\log_{10}$ of the estimated bacterial concentration. In over 2,000 quantitative estimations,

such smears have shown a high degree of internal consistency—progressive increase in estimated concentration with increase of time in progressing infections as well as correlation with clinical indices of severity of infection. They have permitted quantitation of the bacteriologic effect of experimental antibiotic treatment with acceptable standard errors (Table I). In a series of 20 specimens, estimated simultaneously by direct smear and by Petroff-Hauser chamber counts, there was agreement, to the nearest $\log_{10}$, in 18 of the specimens. In a series of replicate smear determinations on 100 specimens the two estimates yielded the same figure in 93 cases. Estimations made on material subjected to successive tenfold dilutions (within the sensitivity range of the procedure) gave the same figure 26 out of 30 times; the successive dilutions were made using the 0.01 ml. loop, starting both with undiluted material and a 10^{-1} dilution made from a swab sample.

TABLE I. EXAMPLE OF DATA OBTAINABLE BY QUANTITATIVE SMEAR ESTIMATES

HOURS AFTER WOUNDING	INFECTED EXPERIMENTAL WOUNDS					
	UNTREATED ANIMALS			PENICILLIN-TREATED ANIMALS		
	N	C	S.E.M.	N	C	S.E.M.
9	15	6.93	±0.23	17	5.30	±0.58
16	13	7.62	±0.40	16	7.12	±0.48
25	6	8.66	±0.26	16	7.68	±0.51

N = Number of animals per group.
C = $\log_{10}$ of mean estimated concentration per milliliter.
S.E.M. = Standard error of mean.

By the use of a number of differential and selective media in the plate estimation method, it is possible within 12 to 24 hours to make a "differential" estimate of the flora in the specimen on the order of the following:

Preliminary culture data indicate the presence in the specimen of the following types of organisms in the approximate per milliliter concentration indicated:

I. Total aerobic organisms	10^7
A. Total gram-positive aerobic	$10^{6.5}$
α-streptococci	10^6
β-streptococci	$10^{6.5}$
B. Total staphylococci	$10^{6.5}$
albus	$10^{6.5}$
aureus	10^6
aureus, presumptively pathogenic	10^6
C. Total gram-negative aerobic	$10^{6.5}$
coliforms	$10^{6.5}$
noncoliforms	10^6
D. Fecal streptococci	10^6
II. Total anaerobic gram-positives	$10^{7.5}$
Lecithinase-producing organisms	$10^{7.5}$

The quantities are estimates only to the nearest whole or half $\log_{10}$ (Fig. 3), which explains why they may not appear to "add up": 1×10^6 plus 1×10^6 equals 2×10^6, which is still "10^6" on such a scale. Even with such approximation, this report is sufficient to imply that the specimen, a wound swab, is from a patient suffering from a severe anaerobic infection, probably clostridial.

In more than 9,000 serial dilution streaked plate estimations the method has shown a high degree of internal consistency (Table II). Determinations of

50 specimens, all with readings in the lower quadrants of the plates, agreed with the results of parallel pour plate determinations ±0.5 log in 48 instances.

TABLE II. EXAMPLE OF DATA OBTAINABLE BY SERIAL DILUTION STREAKED PLATE ESTIMATES

HOURS AFTER WOUNDING	INFECTED EXPERIMENTAL WOUNDS TOTAL ANAEROBIC GRAM-POSITIVE ORGANISMS	
	UNTREATED ANIMALS	PENICILLIN-TREATED ANIMALS
4	3.56 ± 0.24	3.30 ± 0.32
6.25	5.00 ± 0.29	3.45 ± 0.27
9	6.50 ± 0.12	4.70 ± 0.28
12	6.84 ± 0.10	6.20 ± 0.29
16	7.04 ± 0.14	6.40 ± 0.16
25	7.15 ± 0.16	6.85 ± 0.12

Twenty-five animals in each group. Estimates expressed as $\log_{10}$ of mean bacterial concentration per milliliter, ± standard error of mean.

DISCUSSION

The simplicity of both the smear estimations and plate estimations invites ready replication. Even in pour plate counting, a few replications makes for greater validity of the estimate than does meticulous or perfect measuring and dilution of the sample.[1]

For samples taken by swab, replicate sampling is virtually mandatory. A possible exception is in experimental work when a great many determinations are made, and sampling errors can be assumed to "average out"; a great deal of the variance in the data in Tables I and II is accounted for by the fact that only single samples were taken.

For clinical work it would appear wise to have the swab samples taken by laboratory personnel rather than by the clinician; this is not a matter of getting a "better" sample, but *consistent* samples, so that the currency of the quantitative data will have a stable rate of exchange. In the experimental studies for which these methods were developed, sampling times were so scheduled that one individual could make more than 80 per cent of the swabs.

So long as estimates are made on a logarithmic basis, the sampling error does not appear to be affected by the usual variations in the quantity picked up by the swab. Sanford and associates,[2] have reported the standard swab to pick up "110 ± 22 mg., *depending upon the character of the exudate*" (italics added). In infected experimental wounds in goats we have found the pickup to be 170 mg. ± 20 mg. (standard deviation). Shortly after wounding the exudate is bloody, and the mean pickup is 170 mg. Within a few hours a thin serous exudate appears; the mean pickup drops to 140 mg. Later the exudate is seropurulent, with a mean pickup of 170 mg. With late and frankly purulent exudates, the mean pickup is 240 mg. From a limited number of determinations on bloody exudate containing Cr^{51}-tagged red cells,* it appears that the swab *delivers* approximately half of the pickup when it is wrung out in 0.9 ml. of broth. For logarithmic estimations, it appears sound to accept the range of mean delivery (70 to 120 mg.) as resulting in a 10^{-1} dilution in 0.9 ml. of broth.

*Four determinations, kindly performed by Captain Henry M. Wise.

Even when swabs are taken by the same individual, and show close agreement in pickup weight, there is considerable variation between samples. In 100 paired determinations, 56 pairs gave the same reading on the plate scale; 38 differed by one scale reading; 6 differed by two scale readings (1 to 1.5 log).

It is difficult to decide on the basis for calibration of the smear technique; perhaps no calibration is required—the organisms are *there*, they can be seen and counted. The smear technique does not differentiate between viable and nonviable organisms. Plate techniques do not discriminate between single organisms and clumps. For these reasons, smear estimations generally run one log higher than parallel pour plate or serial dilution streaked plate estimations.

For "nearest log" estimations, the dividing line is a decimal scale of "3." If one organism per field represents 10^6 organisms per milliliter in the original specimen, then 3 organisms per field = $10^{6.5}$ organisms per milliliter = "10^7 per milliliter." It is our custom to start the examination in the center of the drop and work out field by field through the edge of the drop, up or down one field, then back to the center for a total of approximately 30 fields. Working with a 10^{-1} dilution, and a field of 100 μ radius, and judging the average or typical field by "sight" and not by actual count, the estimate is made on the following scale:

No organisms seen in		
30 fields (i.e., <3 per 100 fields)	=	$<10^5$
3 per 100 fields to 3 per 10 fields	=	10^5
3 per 10 fields to 3 per field	=	10^6
3 per field to 30 per field	=	10^7
30 per field to 300 per field	=	10^8
300 per field to 3,000 per field	=	10^9
>3,000 per field	=	$>10^9$

Calibration of the serial dilution streaked plate method should be performed in the individual laboratory. For estimates made on growth limited to the upper half of the plate, this is a matter of simple arithmetic: a sample of known size and known dilution has been plated. For estimations on the lower half of the plate, a scale such as that shown in Fig. 3 is determined from actual experience in comparison to pour plate determinations. The actual scale will depend on the technique of streaking, the character of the surface of the plates used, and the character of the material streaked, that is, on the efficiency of pickup and distribution of material from the previously streaked area.

The differential and selective media and incubation technique used as a basis for presumptive or generic identifications in rapid quantitative estimations is a matter of choice for the individual laboratory. Our experience has been limited to the following, which are sufficient to support a report of a bacteriologic "spectrum" such as the example cited above.

Blood agar, aerobic, 37° C.: total aerobic estimate.

Blood azide agar, aerobic, 37° C.: total aerobic gram-positives; differentiation of hemolytic reactions without interference from gram-negative overgrowth.

Salt mannitol agar, aerobic, 37° C.: total staphylococci; differentiation of presumptively pathogenic organisms.[3]

MacConkey's agar, aerobic, 37° C.: total gram-negatives; differentiation of coliforms and noncoliforms.[4]

SF agar, aerobic, 45.5° C.: fecal streptococci.

Blood azide egg yolk agar, anaerobic, 37° C.: total anaerobic gram-positives; differentiation of various types of hemolytic and lecithinitic reactions.

We make the SF agar by adding 30 Gm. per liter of plain agar to SF broth.[5] The blood azide egg yolk agar is based on the medium of Evans.[6] Briefly, it consists of an azide blood agar, to which is added 5 per cent by volume of a 1:1 dilution of sterile egg yolk in saline. The others are standard media.

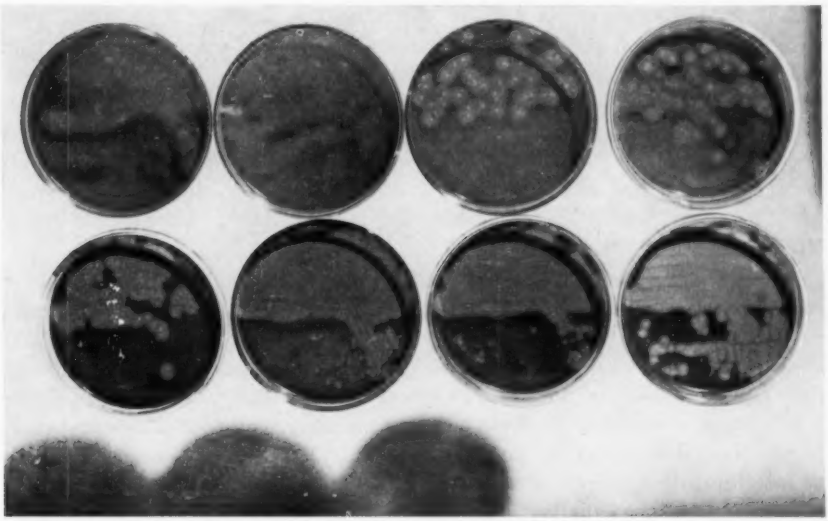

Fig. 4.—Typical readings on serial dilution streaked plates. Blood azide egg yolk medium of Evans, anaerobic incubation. Estimations based on lecithinase-positive colonies only. Top row (left to right): less than 10^2 (no lecithinase-positive colonies, hemolytic streptococci only); 10^2 (single lecithinase-positive colony in upper right of plate); 10^4; 10^5 (there is growth in lower quadrants, but it is evident at a glance that there are less than 1,000 colonies in the upper half of the plate). Lower row (left to right): 10^6, $10^{6.5}$, 10^7, and $10^{7.5}$.

For the method of streaking these plates, to give quantitative estimations at a glance (Fig. 4), and on a single plate, of concentrations within a range of some 7 logs ($<10^3$ to $>10^{7.5}$), I am indebted to Baker and Noyes.[7] Surface plate counting is not new; methods are well known for distributing a sample by tipping the plate, spreading with a glass rod, or dropping from a pipette.[8] Baker and Noyes demonstrated the simplicity and utility of a standard-volume loop, and the principle which I have come to call serial dilution streaking, to make estimations over a range of 5 logs ($<10^4$ to $>10^6$). The plate estimation method described herein is simply a further extension of their principle, for which they deserve the entire credit.

The principle of placing a known quantity of a known dilution of material on a known area of a slide to permit quantitative determinations on stained

smear has been known for over 60 years.[9] It was the basis of the familiar milk count of Breed.[10] The method described here is nothing more than a simplification of some of the steps: use of a loop instead of a pipette, and delivery by the loop to a relatively constant area on the slide, rather than the tediousness of spreading the material over an area 1 cm. square. The "sight reading" of bacterial concentration from such a standard drop was suggested to me by the "pinhead" leucocyte count of Benjamin.[11]

The stained smear technique is more sensitive in the lower ranges of counting than is the chamber method, but it still has a rather high lower limit, for obvious reasons. A 0.01 ml. drop of material containing 1,000 organisms per milliliter will contain on the average only 10 organisms, or about 1 per 100 fields.

Examination of milk by stained smear is a rather simple business. Milk normally contains little proteinaceous debris, and fat is readily cleared by solvents. On the other hand, purulent material and serous or sanguineous exudates have been disappointing subjects for smear examination. Unless they are diluted (which decreases the sensitivity of the method in low bacterial concentrations), smears of such material are often virtually unreadable due to background. However, a method of clearing such smears is available,[12] and the results in our hands have been gratifying.

Neither of these quantitative methods requires much additional time or effort on the part of laboratory personnel. If smears must be made, they may as well be made by a technique which permits quantitative comparisons. Air-drying of smears without heat, which appears to be essential to good clearing, does take an additional amount of elapsed time per smear, but it can easily be worked in as a matter of production-line stages along with other laboratory work. If plates must be streaked, they may as well be streaked with a method which permits quantitative estimations; some additional time is required due to the additional flaming of the loop. However, in comparison to the laboratory effort entailed in making a battery of pour plates for a quantitative unknown, the time required for making one serial dilution streaked plate (or duplicates, or triplicates) is more than compensation. The streak method is admittedly not so accurate, but it is relatively seldom that the 2 or 3 or 4 significant figures in a pour plate count are truly of more biologic significance than the single significant figure of a logarithmic estimate.

SUMMARY

By the use of two simple methods—quantitative preparation of stained smears, and serial dilution streaking of plates of differential and selective media—it is possible to produce quantitative and qualitative bacteriologic data of useful, although approximate, accuracy and specificity within 12 to 24 hours of the time a specimen is taken.

I am grateful to Prof. Kenneth L. Burdon and Miss Arlene Strouse, and to Dr. Howard E. Noyes, Lieutenant Colonel Hinton Baker, and Lieutenant Albert T. Knecht, my tutors and preceptors in bacteriology.

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AN ACCURATE DEVICE FOR THE MEASUREMENT OF DISTANCE ON THE ELECTROCARDIOGRAM AND OTHER PERMANENT RECORDS

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FOR accurately determining the interrelationship of the events in each of the limb and chest leads of the electrocardiogram, it has long been customary to employ a dual-channel recorder. One of the channels is used for recording each of the limb and chest leads in succession while the other channel is used as a reference lead. The reference lead then serves as a satisfactory time index relative to which the events in the other leads may be measured.

To facilitate electrocardiographic measurement, a number of devices both mechanical and optical have been described. The majority of these consist of rulers or other scales inscribed so as to permit the direct conversion of distance into time.[1] Others consist of caliper-like instruments in which the distance between fixed and movable indicator marks is read on an attached scale.[2, 3] Some of these permit alteration of the scale factor and are adaptable to the use of verniers for increased accuracy. None of these, however, was readily adaptable to the present problem involving the measurement of a large number of simultaneous recordings. This difficulty has been overcome by the device herein described which has been successfully used on a large number of records collected recently by one of us (R. M. P.).

MATERIALS AND METHODS

Method of Construction.—The device consists of an indicator line, the movement of which is registered by an accurate dial indicator; a threaded knob for advancing the indicator line smoothly; and a nonslip base for holding the entire device firmly on top of the electrocardiogram. This is illustrated in Fig. 1.

A dial indicator graduated in units of 0.01 mm. with a total traverse of 25 mm.† was fastened in a convenient position to a stand constructed of 5/16 inch aluminum stock. Not visible in the figure is a small aluminum support projecting up from the base of the stand to which the lug at the back of the dial indicator was attached by a suitable screw. A bronze bushing 3/4 inch long was force-fitted into the upright portion of the stand coaxial with the

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actuator rod of the dial indicator. The bushing was tapped to receive a 5/16-14 threaded brass rod with a knurled knob at one end. This threaded rod turns smoothly in the bushing for a distance sufficient to advance the dial indicator the full 25 mm. An aluminum turning was drilled to receive the extension of the right-hand end of the actuator arm. (This extension is available with the dial indicator or can be made from a suitable steel rod.) A plastic indicator was attached to the face of the aluminum turning. A single line 6 cm. long was inscribed on the underside of the plastic and filled with red pigment to contrast with the ECG. This line serves as the indicator. Final adjustment of the height of the indicator line was simplified by using slots in the plastic at its point of attachment to the aluminum turning. A carrying knob was attached at the right-hand end of the stand to facilitate lifting without damaging the actuator rod. A sheet of firm rubber, 1/8 inch thick, was cemented to the bottom of the aluminum base.

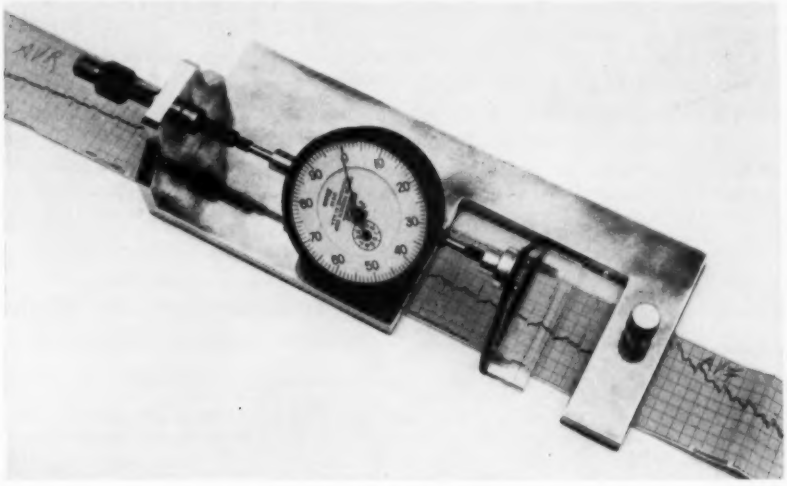

Fig. 1.

Method of Operation.—The instrument is placed on the record to be measured so that the indicator line is parallel to the time line of the record, i.e., at right angles to the direction in which the record moved through the recorder. Its operation involves only turning the knurled knob to advance the actuator rod of the dial indicator and, with it, the indicator line. Distances are then read directly from the face of the dial indicator. Note that the face of the dial indicator rotates so that zero may be placed under the pointer for any convenient location of the line. The indicator line is long enough to cover both channels of the average dual channel recording and to permit setting the dial at zero with the line correctly located relative to the constant or timing channel.

The process of multiplication required to convert the measured distances to time can be simplified by the use of a conversion table or by the use of a dial face graduated in milliseconds.

DISCUSSION

This device lends itself well to the measurement of many types of medical recordings including electroencephalograms, electroretinograms, pulse and pressure recordings, and ballistocardiograms. It may also find application in the measurement of spectrographic and other photographic plates as well as of paper

electrophoresis patterns. If necessary, dial indicators with longer traverse or of greater sensitivity may be employed.

Accuracy is limited only by parallax and by the accuracy of the dial indicator selected. The former is held to a minimum by the location of the indicator line in close proximity to the record being measured. The accuracy of available dial indicators far exceeds that necessary for the most exacting measurements. The type indicator employed here is routinely tested at 0.25 mm. intervals over its entire range for compliance with the commercial tolerance of plus or minus one scale division (0.01 mm.) as specified by the National Bureau of Standards.[4] It is clearly more accurate and linear than the most reliable available rule and has proved so in use. In our experience the limiting factor has always been the linearity of the record itself.

SUMMARY

The construction and operation of an instrument for the accurate measurement of electrocardiograms and other records is described.

We wish to thank the Department of Radiology and, in particular, Norman R. Horwitz, Wayne State University, for advice and the use of their facilities in the construction of this instrument.

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INFLUENCE OF FUNGUS STRAIN AND LAPSE OF TIME IN EXPERIMENTAL HISTOPLASMOSIS AND OF VOLUME OF INOCULUM IN CRYPTOCOCCOSIS UPON RECOVERY OF THE FUNGI

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THE etiologic diagnosis of a mycotic infection requires the isolation and identification of the fungus. Fungi may be isolated by direct culture or by animal inoculation of pathologic materials. In some instances of proved mycoses, both methods of obtaining cultures may fail.

The difficulties sometimes encountered in obtaining a positive culture led us to investigate two factors influencing the isolation of pathogenic fungi. These were: (1) the effect of increasing the time interval between inoculation of a fungus into mice and sacrifice of the animals for its recovery in culture from the organs, and (2) the influence of increasing the volume of cerebrospinal fluid used for culture on the isolation of *Cryptococcus neoformans* from patients with cryptococcal meningitis.

MATERIALS AND METHODS

Female Swiss white mice (NIH general purpose strain) weighing 17 to 20 grams were used in all experiments. Mice were infected with *Histoplasma capsulatum*, strain 6624, or with *C. neoformans*, strain 1148. Both of these fungi have been used in many experimental studies and have maintained a constant virulence for mice during many serial transfers in culture. However, strain 6624 no longer sporulates freely in culture and may be atypical in other unrecognized characteristics. In later experiments, 3 strains of *H. capsulatum* (F 9961, F 9965, and F 9673) recently isolated from patients in the Clinical Center, National Institutes of Health, and one strain (F 9671) recently isolated from soil on premises where a patient with fatal histoplasmosis had been exposed were converted to the yeast phase and subjected to the same tests.

Yeast phase cultures of *H. capsulatum* were harvested in saline from cysteine blood agar slants after incubation at 37° C. for 48 hours. The suspension was diluted in saline so that each mouse received an inoculum containing 10, 10^2, 10^3, 10^4, 10^5, or 10^6 cells. Hemocytometer counts were used to estimate the number of organisms in the concentrated suspensions. The accuracy of the more dilute suspensions was occasionally checked by culturing in duplicate 1 ml. of the saline suspension on 20 ml. of Sabouraud's agar in a 125 ml. flask.

C. neoformans was harvested in saline from a Sabouraud's agar slant after incubation for 24 hours at 30° C. Dilutions and hemocytometer and colony counts were done in the same manner as for *H. capsulatum*.

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Each of the cultures studied was injected in the indicated concentrations intraperitoneally or intravenously into separate groups of 10 mice each. Two animals from each group were sacrificed at various intervals from 2 to 14 weeks following infection. The liver and spleen were removed and a portion of each was placed separately on a Sabouraud's agar slant, crushed with a stiff wire loop, drawn back and forth across the slant several times, and left on the agar. This method of culturing tissues from experimental mice (rather than the more quantitative method of grinding and diluting weighed portions of tissues) was used because it is the method commonly used in this laboratory for examination of mice inoculated with pathologic tissues or exudates. It was the efficiency of this method which was being tested. All slants were incubated at 30° C. and examined for growth after 1, 2, and 4 weeks.

The effect of increasing the volume of cerebrospinal fluid used in direct cultures on the recovery of Cryptococcus was studied in 7 patients with suspected or proved cryptococcal meningitis. A specimen consisting of 4 to 8 ml. of spinal fluid was cultured in each experiment. The fluid was distributed between test tubes containing 5 ml. of Sabouraud's glucose agar "slants" and Erlenmeyer flasks (125 ml.) containing 20 ml. of agar. Either 0.1 or 0.25 ml. of spinal fluid was pipetted into each tube, and 0.5, 1.0, or 2.0 ml. was pipetted into each flask. The tubes were kept semihorizontal until the spinal fluid had dried on the slant. In addition, a 2 ml. sample was centrifuged at 3,000 r.p.m. for 20 minutes and 0.1 ml. of the "sediment" was pipetted in duplicate onto the slants. All tubes and flasks were incubated at 30° C. and were observed for 3 months before being considered sterile.

TABLE I. RECOVERY OF AN OLD LABORATORY STRAIN OF H. CAPSULATUM FROM THE LIVERS AND SPLEENS OF MICE WITH INFECTION OF INCREASING DURATION FOLLOWING INTRAPERITONEAL INOCULATION. AT EACH TIME INTERVAL MICE RECEIVING FROM 10 TO 10^5 CELLS OF H. CAPSULATUM (F 6624) ARE GROUPED TOGETHER SINCE THE RESULTS WERE SIMILAR FOR ALL SIZES OF INOCULA USED

WEEKS*	NUMBER OF COLONIES PER SLANT					TOTAL CULTURES (LIVER AND SPLEEN)
	0	1-3	4-15	16-49	50	
2	2†	0	1	11	22	36
4	4	4	13	8	7	36
6	3	6	4	3	0	16
8	29	5	2	0	0	36
10	16	0	0	0	0	16

*Duration of animal infection prior to sacrifice.

†The two sterile cultures represent the liver and spleen of an animal inoculated with 10 yeast cells.

RESULTS

Recovery of Fungi After Animal Inoculation.—

Histoplasma capsulatum: The results of the first two experiments, using mice infected intraperitoneally with an old, nearly sterile strain of *H. capsulatum* (6624) are summarized in Table I. Mice receiving all sizes of inocula from 10 to 10^5 yeast cells are recorded together in the table, since the final results were generally similar in all groups.

Cultures of liver and spleen were positive in 17 of 18 mice sacrificed 2 weeks after intraperitoneal infection, and the majority showed heavy growth. The cultures from one animal which received an estimated 10 organisms were negative.

Six weeks after infection, 7 of 8 mice still yielded positive cultures from either the liver or spleen, but there was a definite decrease in the number of colonies on each slant.

Only 7 of 36 cultures of liver and spleen taken from 18 mice sacrificed 8 weeks after intraperitoneal infection showed growth of *H. capsulatum*, and these yielded less than 16 colonies per slant. All cultures from animals sacrificed 10 weeks after infection were sterile.

Mice infected intravenously with 10 to 10^5 cells of strain 6624 showed a similar progressive decrease in the proportion of positive cultures, although the decline in recoverable fungi was less striking than seen after intraperitoneal infection.

Results obtained were different when 4 recently isolated strains of *H. capsulatum* were tested under similar conditions. Groups of 10 mice were injected intraperitoneally with 10^2, 10^4, or 10^6 cells of each strain. The recorded degree of recovery of Histoplasma from these mice (Table II) is based upon the average colony count in 4 agar slants (2 spleen and 2 liver) from each mouse. In two of the strains, F 9661 and F 9673, there was no observed decrease in the number of colonies growing in cultures with either small inocula or lapse of time up to 10 weeks. In the other two strains, there was a decrease in number of colonies after the lapse of 4 weeks in mice infected with 10^2 and 10^4 cells, although the lower level was sustained at the tenth week. An inoculum of 10^6 cells of F 9671 was lethal and no mice survived for testing at 10 weeks. Similarly, 10^6 cells of F 9665 and of F 9673 killed the last of the mice before the 8 weeks' testing period.

TABLE II. RECOVERY OF 4 TYPICAL, RECENTLY ISOLATED STRAINS OF H. CAPSULATUM AT VARYING TIME INTERVALS AFTER INTRAPERITONEAL INOCULATION AND AT 3 LEVELS OF INOCULA

STRAIN	INFECTING DOSE	2 WEEKS	4 WEEKS	8 WEEKS	10 WEEKS
F 9661	10^2	4+*	4+	4+	4+
	10^4	4+	4+	4+	4+
	10^6	4+	4+	4+	4+
F 9665	10^2	4+	3+	2+	1+
	10^4	4+	4+	2+	1+
	10^6	4+	4+	X†	X
F 9671	10^2	4+	3+	3+	3+
	10^4	4+	2+	2+	3+
	10^6	4+	4+	2+	X
F 9673	10^2	4+	4+	4+	4+
	10^4	4+	4+	4+	4+
	10^6	4+	4+	X	X

*Key: 4+ represents 50 or more colonies per slant; 3+, 16 to 49 colonies; 2+, 4 to 15 colonies; and 1+, 1 to 3 colonies.

†X = no surviving mice for examination.

Since the results with the 4 recently isolated strains did not parallel those observed with the old laboratory strain, all 5 strains were retested simultaneously. Cultures were harvested at 48 hours by suspending cells in cold saline containing 0.1 per cent cysteine. Mice were infected intravenously with 10,

10^2, or 10^3 yeast cells of Histoplasma. At autopsy, the spleen was split longitudinally and planted on two slants. A cube of liver approximately 5 mm. on a side was similarly divided between two slants.

Cultures were graded 1 to 4 plus as before. Later, in tabulating results, it was found desirable to estimate the total number of colonies isolated from each mouse and median values were assigned to each of the 3 lower grades (1+ = 2, 2+ = 9, 3+ = 33) and 4+ was valued at 125. Using these estimates, Table III was constructed to show the approximate number of Histoplasma colonies isolated in the entire experiment. Each figure in the Table represents the estimated number of colonies on 8 slants inoculated with spleens and livers of 2 mice.

TABLE III. ESTIMATED TOTAL NUMBER OF COLONIES OF HISTOPLASMA ISOLATED AT THE INOCULATION LEVELS AND AFTER TIME INTERVALS INDICATED. EACH NUMBER REPRESENTS ESTIMATED NUMBER OF COLONIES ON 8 SLANTS INOCULATED FROM SPLEENS AND LIVERS OF TWO MICE

STRAIN	INFECTING DOSE	2 WEEKS	4 WEEKS	6 WEEKS	10 WEEKS	14 WEEKS
6624	10	0	0	0	0	0
	10^2	46	84	132	66	2
	10^3	130	168	17	4	0
F 9661	10	522	28	15	33	8
	10^2	1000	167	324	13	2
	10^3	608	88	30	26	226
F 9665	10	400	88	2	0	0
	10^2	553	40	4	2	0
	10^3	560	383	9	13	2
F 9671	10	345	106	26	6	36
	10^2	246	86	33	6	31
	10^3	415	128	2	57	44
F 9673	10	536	99	147	134	86
	10^2	540	48	168	111	59
	10^3	632	17	79	113	40

In general, in the mice inoculated intravenously, there is a much more apparent decrease in the number of colonies recovered than in mice inoculated intraperitoneally with the same strain of Histoplasma. With the exception of strain 6624, there was a decrease in the number of colonies at the fourth weeks' sampling. However, in 5 of the 15 pairs of mice killed after 6 weeks, there was a rise in the number of colonies. Strains F 9671 and F 9673 and 10^3 cells of F 9661 produced a high level of infection up to 14 weeks. However, at 14 weeks 4 of 15 pairs of mice did not yield cultures. No cultures were recovered from any of the mice inoculated with 10 cells of strain 6624, and these 10 mice are omitted from consideration in the following two paragraphs.

Data from which Table III was derived show that 3.5 per cent of the cultures from mice killed 2 weeks after inoculation were sterile. The number of sterile cultures rose to 22 per cent in 4 weeks, 40 per cent in 6 weeks, 50 per cent in 10 weeks, and 59 per cent in 14 weeks.

Large differences in ability to survive and to produce chronic infections in mice were noted among the 5 strains tested. Thus, at 14 weeks, 94 per cent

of the cultures for 6624 were negative; 96 per cent for F 9665, 58 per cent for F 9661, 35 per cent for F 9671, and 25 per cent for F 9673 were likewise negative. For all 5 strains at all of the 5 sampling periods, 234 of 600 cultures (39 per cent) were negative and 95 of these (40.6 per cent) were from mice inoculated with an estimated 10 cells. Of the 360 cultures made from mice receiving strains F 9661, F 9671, and F 9673, 86 (24 per cent) were negative and 32 of these (37 per cent) were from mice inoculated with an estimated 10 cells.

Recovery of Cryptococcus neoformans: After mouse inoculation no decline in liver and spleen fungal populations with lapse of time was noted following intraperitoneal infection with *C. neoformans*. All cultures of liver and spleen remained strongly positive up to 10 weeks after intraperitoneal infection with 10 to 10^5 cells. There was no evidence that delay in sacrifice of the mice resulted in any change in the number of mice with positive cultures or in the number of colonies per positive slant.

TABLE IV. INFLUENCE OF VOLUME OF SPINAL FLUID CULTURED ON THE PROPORTION OF POSITIVE CULTURES FROM THREE PATIENTS WITH CRYPTOCOCCAL MENINGITIS

PATIENT	NUMBER OF LUMBAR PUNCTURES	VOLUME OF SPINAL FLUID PER TUBE OR FLASK (ML.)	NUMBER OF POSITIVE CULTURES PER TOTAL NUMBER INOCULATED OF CULTURES	COLONIES PER POSITIVE CULTURE
D. K.	3	0.1	1/8	1
		0.25	0/8	–
		0.5	1/4	1
		1.0	3/5	1,3,4
W. G.	5	0.1 to 0.25	0/20	–
		2.0	1/2	2
J. N.	4	0.1	0/14	–
		0.25	2/8	1,1
		0.5	1/6	1
		1.0	4/5	1,1,2,2
		2.0	3/5	1,1,3

Recovery of Cryptococcus neoformans From Cerebrospinal Fluid by Direct Culture.—Four of the seven patients studied had many viable organisms per milliliter of spinal fluid as determined by colony counts on Sabouraud's agar. In these patients, as little as 0.05 ml. of spinal fluid gave positive cultures.

In the other 3 patients, the results of culture were influenced significantly by the size of the spinal fluid sample. The results of several spinal fluid studies on each of these patients are summarized in Table IV. The spinal fluid of each of these patients had on the average approximately one viable cell of *C. neoformans* per milliliter, as determined by colony count on Sabouraud's agar. It is clear from Table IV that, when the concentration of viable organisms in the spinal fluid was low, cultures were more likely to be positive if larger volumes of fluid were used. This was true regardless of whether the entire sample was placed in 1 or 2 flasks, or whether it was distributed among many tubes. On several occasions, only one colony of *C. neoformans* grew from

a total sample of 4 ml. of spinal fluid. In none of the 3 patients was the organism isolated by culturing a total of 0.2 ml. of "sediment" from 2 ml. centrifuged at 3,000 r.p.m. for 20 minutes.

DISCUSSION

Previous studies by Salvin[1] and quantitative tests by Rowley and Huber[2] indicated that tissue titers were maximal 14 days after infection with *H. capsulatum* and progressively decreased thereafter. Our studies confirm these observations and demonstrate differences among strains of Histoplasma and one fallacy of using old atypical laboratory strains of fungi in investigations of this type.

When infected tissues or body fluids from patients suspected of *H. capsulatum* infection are injected intraperitoneally into mice, the time interval between animal injection and sacrifice of the mice for culture may be of great importance. In the present studies, best recovery of fungi was obtained with one old laboratory strain and two more recently isolated strains when the animals were sacrificed 2 weeks after infection. With these strains, as the duration of time following animal inoculation was increased, the number of animals demonstrating infection, or the number of colonies isolated from infected mice decreased, suggesting that mice spontaneously eliminate or markedly diminish *H. capsulatum* infections. Two other strains possessed a greater ability to survive and produce a chronic infection in the mouse. No decline with time in isolations of fungi was noted in experimental *C. neoformans* infections.

It is suggested that in diagnostic studies several mice be sacrificed 2 weeks after intraperitoneal injection of tissues or body fluids where *H. capsulatum* infections are suspected. This would provide optimum conditions for the isolation of some strains of *H. capsulatum* and would also be satisfactory for the recovery of *C. neoformans*. The optimum time of sacrifice for isolation of other pathogenic fungi (including other strains of *H. capsulatum*) has not been adequately studied. Until this time is known, additional mice should be sacrificed 4 to 8 weeks after injection.

When mice are inoculated with soil suspensions in studies of saprophytic sources of Histoplasma and other pathogenic fungi, other cogent considerations may dictate a longer time interval between inoculation and sacrifice. Saprophytic bacteria and fungi present in such inocula may survive for some time. Unless mice are allowed to live until such saprophytes have been destroyed, growth of pathogens may be inhibited or obscured. Therefore, an interval of 4 to 6 weeks between inoculation and sacrifice is customarily allowed as an acceptable compromise in studies of this type.

Three of the 7 patients with suspected or previously proved cryptococcal meningitis had negative spinal fluid cultures when small volumes were used for direct culture. In each case, Cryptococcus was isolated when the size of the sample used for culture was increased. It is probable that the value of culturing large samples applies equally well to the isolation of other species of fungi and also to other body fluids and exudates.

Therefore, when conventional cultures for fungi are negative in a patient suspected of harboring a mycotic infection, it is suggested that larger volumes of fluid be cultured. As the size of the sample is increased, it is important to increase the volume of culture medium used, since a high blood or exudate to medium ratio may inhibit the growth of some microorganisms. At least 20 ml. of blood, urine or exudate, and a minimum of 5 ml. of spinal fluid should be used in order to increase the likelihood of obtaining positive cultures.

SUMMARY

The present experiments show that the time interval between inoculation and sacrifice was important in the recovery of some strains of *H. capsulatum* from liver and spleen of mice used in experimental studies. The optimum interval was 2 weeks. Increasing the interval to 8 to 14 weeks decreased the proportion of positive cultures of certain strains. A uniform decrease was not apparent in some recently isolated strains. No decrease in positive cultures was noted upon increasing the interval between inoculation and sacrifice after infection with *C. neoformans*. When mouse inoculation is used as a method of diagnosing suspected fungal disease, it is suggested that several mice be sacrificed 2 weeks after intraperitoneal injection of specimens possibly infected with fungi, and that additional mice be sacrificed 4 to 8 weeks after inoculation.

Evidence is also presented indicating that culture of a large volume of spinal fluid is an important factor in the isolation of *C. neoformans* from some patients with cryptococcal meningitis. It is suggested that at least 20 ml. of blood, urine or exudate, and a minimum of 5 ml. of spinal fluid be cultured when conventional cultures for fungi are negative in a patient suspected of harboring a mycotic infection.

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A PRACTICAL METHOD FOR THE SEPARATION OF LYMPHOCYTES FROM GRANULOCYTES

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MANY methods have been described for the isolation of leukocytes from whole blood, as well as for the separation of peripheral blood lymphocytes and granulocytes.

Jago[1] has recently reviewed the literature so that it is only necessary to mention a few of the more important and latest contributions.

Kline[2] modified Ottesen's method and used a "Lucite swimmer." Following centrifugation, a well-defined layer of cells, containing the lymphocytes, is present within the "swimmer's" central capillary. Careful aspirations resulted in lymphocyte fractions above 90 per cent in purity.

Jago[1] described a method based on the differing densities of lymphocytes and granulocytes and employing sedimentation and centrifugation. In her experiments the mean percentage yield of lymphocytes was 32 ± 22, the mean percentage purity of lymphocytes 99 ± 2.2.

Philippu[3] used the method described by Szilard and separated the different types of leukocytes by centrifugation of the leukocyte suspension in a Wintrobe tube. He reported a top layer consisting of 92 to 96 per cent lymphocytes and a bottom layer of 90 to 98 per cent granulocytes.

This laboratory has long been engaged in the studies of leukocyte physiology.[4-6] As part of these investigations, it has become important to separate lymphocytes and granulocytes of the peripheral blood with a sufficient yield to enable us to perform desoxyribose nucleic acid (DNA) extractions. Accordingly, a simple method has been developed which allows good separation and yield.

MATERIALS AND METHODS

Methods.—Initial separation of W.B.C. from R.B.C. is performed with the use of a dextran sedimentation method as mentioned in the articles by Skoog and Beck.[7, 8] Lymphocytes and granulocytes, differing in density, are then separated by centrifugation.

Blood is withdrawn in siliconized syringes from the jugular vein of a dog. Twenty-five milliliter aliquots of this blood are added to 10 ml. of 6 per cent dextran* in saline and 0.5 ml. of 5 per cent Sequestrene† in distilled water as an anticoagulant. Following sedimentation of the agglomerated erythrocytes, which takes approximately 20 to 25 minutes at room temperature, the supernatant containing the leukocytes is pipetted into 50

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*Dextran with an average molecular weight of 228,000; supplied by the R. K. Laros Company, Bethlehem, Pa.

†Sequestrene disodium (disodium-ethylenediaminetetraacetate dihydrate), Alrose Chemical Company, Providence 1, R. I.

ml. centrifuge tubes. This is then centrifuged for 30 minutes at 1,800 to 2,000 r.p.m. in a size 2 International centrifuge in a coldroom at 4° C.

The supernatant plasma-dextran solution is carefully withdrawn to approximately 0.5 cm. above the sediment. All sediments are pooled together and resuspended in the plasma-dextran supernatant or normal saline so that a final suspension of 40,000 to 50,000 leukocytes per cubic millimeter is obtained.

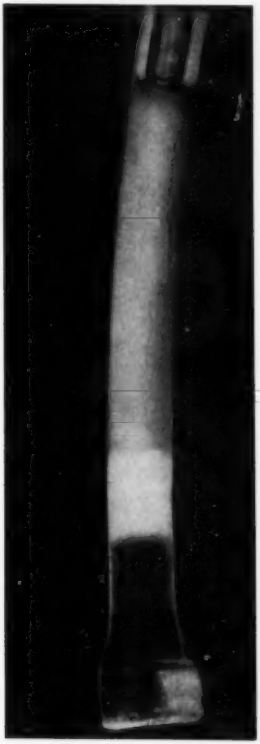

Fig. 1.—Lymphocyte-granulocyte separation in plastic tube. Top white layer is lymphocyte-rich, middle layer is granulocyte-rich, and bottom layer contains red cells with very few leukocytes.

The concentrated leukocyte suspension is centrifuged in plastic tubes especially developed for that purpose. Tygon tubing,* outer diameter ¼ in., inner diameter 3/16 in., and wall thickness 1/32 in., is cut into 5 in. lengths and sealed at one end with a Dobeckmun Super Sealer.† These plastic tubes with a capacity of 2 ml. are filled with the suspension by means of a Wintrobe hematocrit pipette. To prevent bending during centrifugation, the plastic tubes are placed within small Lusteroid tubes.

After centrifugation for 15 minutes at 1,800 to 2,000 r.p.m., the sediment shows 3 distinct layers (Fig. 1). The top layer is white and contains lymphocytes and platelets. The second layer is pink and granulocyte-rich. The bottom layer, dark red in color, consists mainly of erythrocytes. The layers are separated by the application of surgical hemostats and cut with a razor blade at the junction of the layers.

*Tygon tubing is a product of U. S. Stoneware Company, Plastic & Synthetic Division, Akron, Ohio.

†Dobeckmun Super Sealer, Dobeckmun Company, Cleveland 1, Ohio, and Berkeley 2, Calif.

During the separation procedure, leukocyte and differential counts were done on the various supernates and cell suspensions. Volumes were recorded in order that percentage purity and yields could be calculated.

TABLE I. RESULTS OF LYMPHOCYTE-GRANULOCYTE SEPARATIONS

Dog No.		100-131	82-132	157-142	SUMMARY
No. of separations		12	12	1	25
Mean per cent yield of leukocytes		86.9	78.4	90.2	82.9
Range		67.4-100	64.5-93		64.5-100
Lymphocyte-rich samples	Mean per cent yield of lymphocytes from whole blood	77.1	53.6	100	66.7
	Range	24.8-100	15.2-100	–	15.2-100
	Mean per cent yield of lymphocytes from supernatant	83.8	64.5	76.4	74.3
	Range	39.2-100	32.8-100	–	32.8-100
	Mean per cent purity of lymphocytes	72.6	68.8	62	70.4
	Range	66-82	42-85	–	42-85
	Mean per cent granulocyte contamination	25.3	23.8	38	25.1
	Range	16-31	11-50	–	11-50
Granulocyte-rich samples	Mean per cent yield of granulocytes from whole blood	69.8	68.1	31.3	67.4
	Range	44.9-90.1	48.3-88.2	–	31.3-90.1
	Mean per cent yield of granulocytes from supernatant	76.8	86.8	38.6	80.1
	Range	55.8-96.5	71-9-100	–	38.6-100
	Mean per cent purity of granulocytes	76.4	74.8	79	75.7
	Range	58-86	66-83	–	58-83
	Mean per cent lymphocyte contamination	23.3	18.9	21	21.1
	Range	13-38	11-26	–	11-38

RESULTS AND DISCUSSION

The results obtained in 25 separations are shown in Table I.

Many of the methods for lymphocyte-granulocyte separation described in the literature result in a high degree of purity of particular cell preparations. However, the yield of lymphocytes is usually quite poor. The most recently reported technique[1] resulted in a mean per cent yield of lymphocytes of 32 ± 22. With the method described in this paper, the mean per cent yield of lymphocytes was 66.7.

Another advantage of this method is that it is possible to reinfuse the erythrocytes after saline washing. This becomes an important consideration when the test animal must be subjected to large and multiple bleedings.

Since this method was developed in the course of DNA-P^{32} studies in which the DNA is extracted from white cells employing the Simmons technique,[5] platelets containing little or no DNA present no problem. Similarly, the minimal erythrocyte contamination is of no consequence.

Following the determinations of DNA-P^{32}, specific activity for the two cell fractions and, knowing the differential counts, lymphocyte and granulocyte DNA-P^{32} activity are then readily calculated using simultaneous equations.

The use of the plastic Tygon tubes for the final step of the separation has the advantage of increased yield of the cell fractions, reduced contamination by cells from adjoining layers, and diminished loss of cells with handling. Ingram and associates[9] have also employed flexible plastic tubing for the concentration of leukocytes from small samples of blood.

Smears of the various fractions demonstrate that morphologically there appears to be little or no change in cell structure. A slight increase in disintegrated cells is usually noted.

SUMMARY

1. A method is described for lymphocyte-granulocyte separations of the peripheral blood of dogs.

2. Leukocyte suspensions are obtained from whole blood with a modification of the dextran method. The leukocytes, after suitable resuspension, are separated into lymphocyte-rich and granulocyte-rich fractions by centrifugation in special plastic tubes.

3. In 25 separations in a test series of 3 dogs, the mean percentage yield of lymphocytes was 66.7, with a mean percentage purity of lymphocytes of 70.4. The mean percentage yield of granulocytes in the granulocyte-rich specimen was 67.4, with a mean percentage purity of granulocytes of 75.7.

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PREPARATION OF A HUMAN PLASMA FRACTION RICH IN PROTHROMBIN, PROCONVERTIN, STUART FACTOR, AND PTC AND A STUDY OF ITS ACTIVITY AND TOXICITY IN RABBITS AND MAN

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IT HAS been known since the work of Quick in 1937[1] that the intravenous injection of normal rabbit plasma transitorily corrects the prolonged prothrombin time of rabbits that have been fed spoiled sweet clover hay, and that no such correction is obtained following the injection of normal rabbit serum. Since the identification of the toxic principle in spoiled sweet clover hay as a coumarin compound by Stahmann and associates[2] in 1941, others[3, 4] have raised the prothrombin level of coumarinized animals by the injection of prothrombin-rich animal plasma fractions.

Recently the intravenous administration of human plasma fractions rich in antihemophilic factor B (AHF-B, PTC)[5, 6] or proconvertin[7, 8] has been reported in patients congenitally deficient in one or the other of these factors essential for normal hemostasis. The present paper describes (1) the preparation of a human plasma fraction hereinafter referred to as PPB, containing prothrombin, the proconvertin complex (see below), and AHF-B (PTC) in high concentration, (2) its intravenous administration to rabbits made deficient in all these factors by prior administration of a coumarin derivative, and (3) the results of preliminary trials of this product in man. Such a fraction could have therapeutic value not only in the conditions in which vitamin K_1 is of no value such as congenital single deficiencies of these factors[9-16] and in acquired combined deficiencies occurring in hepatocellular insufficiency,[17] but also in conditions in which parenteral vitamin K_1 has a beneficial effect (coumarin drug overdosage,[18] biliary obstruction,[17] and prolonged antibiotic therapy[17]). A plasma fraction would raise the levels of the factors in question immediately whereas the vitamin K_1 effect is delayed.

MATERIALS AND METHODS

Thromboplastin was an acetone extract of human brain; buffer was veronal acetate, pH 7.4. All coagulation factors were assayed by methods previously described by one of us with M. J. Larrieu.[19, 20] What was formerly called "specific assay for proconvertin" utilizing Seitz-filtered bovine plasma as substrate plus calcium and tissue thromboplastin

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will here be referred to as "proconvertin complex" assay, for it has become apparent[16] that a deficiency of proconvertin (factor VII, SPCA precursor[14, 21]) *or* of Stuart factor will give a prolonged clotting time using this substrate.

Stuart factor was assayed by our modification of Bachmann's[22] method: to 0.1 ml. bovine plasma filtered once through a 20 per cent asbestos pad (J. C. Carlson, Ltd.) is added 0.1 ml. of the plasma or fraction to be tested, diluted 1:10 in buffer; 0.1 ml. cephalin[23] diluted 1:30; 0.1 ml. Russell viper venom (Stypven; Burroughs Wellcome) diluted 1:50,000; and 0.1 ml. 0.025 M $CaCl_2$. Stypven concentrations up to 1:10,000 were also found satisfactory. Cephalin and Stypven dilutions were stable at +1° C. for at least one week. Independently a similar test has recently been developed by Sise and associates.[24] Our modification includes the use of the mixed soybean phosphatide preparation Asolectin (Associated Concentrates), 0.1 per cent in buffer, instead of cephalin, as Asolectin appears more stable on storage. Nine-month-old plasma (stored at −20° C.) from M. M., a patient with known congenital Stuart factor deficiency,[22, 25] kindly supplied by Drs. J. Bernard and J. Caen and studied in this laboratory by Dr. M. J. Larrieu, clotted in 90 seconds in this assay system, whereas fresh plasma from Y. G., a patient with known congenital proconvertin deficiency previously reported by one of us,[26] clotted in 20 seconds, as did fresh or stored normal plasma.

The plasma of Y. G. was also used as substrate in a one-stage test[27] of the content of proconvertin in some fractions. For calculation of percentages of all factors, appropriate dilutions in buffer were tested. Protein determinations were done by the biuret method.

Preparation of Fraction PPB.—Ten lots were made by the following procedure which, with minor variations, proved to be the most satisfactory: 400 ml. of blood from each fasting donor were collected under sterile conditions through a plastic column containing 70 Gm. of the cation exchange resin Amberlite IR 120 in the sodium cycle, and into silicone-coated 500 ml. bottles containing 10 ml. of 0.01 to 0.02 M trisodium citrate. The bottles were then centrifuged at +5° C. and 2,600 r.p.m. for 30 minutes. The erythrocytes were ready for therapeutic use as "packed cells" as soon as the plasma was sterilely suctioned off into 500 ml. bottles containing an amount of tricalcium phosphate (Baker, "Analyzed Reagent") such that the latter's concentration in plasma was 0.3 to 0.6 per cent. This adsorbant was chosen as having the highest absorptive capacity per unit weight of several tested (tricalcium phosphates BLB, Fisher, Merck, and Prolabo; barium sulfates Merck and Prolabo). Adsorption was performed by mechanical to-and-fro agitation of these bottles for 10 minutes at room temperature. The bottles were then centrifuged at +5° C. and 2,600 r.p.m. for 30 minutes. The adsorbed plasma was decanted and used as source for the preparation of sterile fibrinogen and AHF-A. The adsorbent was washed once with a volume of normal saline equal to that of the original plasma, and the wash was discarded after centrifugation at 2,600 r.p.m. for 30 minutes. Elution was carried out at room temperature on a magnetic agitator for 15 minutes, the eluant being 1/20 of the original plasma volume of 0.18 M trisodium citrate at pH 8.0. These appeared to be the optimal molarity and pH values, although elution was quite satisfactory with 0.05 to 0.3 M trisodium citrate at pH 5 to 10. Alternatively, two elutions with 1/40 of the plasma volume were pooled, thereby increasing the yield of coagulation factors eluted.

After centrifuging (International refrigerated centrifuge) at 4,000 r.p.m. for 30 minutes, the eluate, which was a clear pale-yellow solution, was further concentrated and cleared of its high citrate content by dilution with an equal volume of distilled water and precipitation at pH 5.2 from a final ethanol concentration of 25 per cent at −5° C. The precipitate, after centrifugation at −5° C., was resuspended in 0.1 M glycine containing 1/10 volume 0.1 M trisodium citrate, the pH was adjusted to 7.0 to 8.0 and the fraction was shell-frozen and lyophilized. Such lyophilized products did not develop thrombic activity over 5 months' time. Prothrombin concentrations were 110 to 220 times that of normal

plasma per milligram of protein. The dry powder was redissolved in distilled water immediately before injection into the rabbits. Solutions injected had a prothrombin concentration 50 to 100 times that of normal plasma per volume. The final average prothrombin yield of the lyophilized fraction was 81 per cent, the range being 46 to 124 per cent. Yields and concentration of each factor at each step of production are listed in Table I. Traces of proaccelerin, fibrinogen, and antihemophilic factor (AHF-A) were also present.

TABLE I. YIELD AND CONCENTRATION OF COAGULATION FACTORS AT EACH STEP IN THE PREPARATION OF HUMAN PLASMA FRACTION "PPB"

STEP	FACTOR	YIELD		CONCENTRATION PER MG. PROTEIN*	
		RANGE (%)	AVERAGE (%)		
Adsorption (per cent adsorbed)	Prothrombin	93-97	95		
	Proconvertin complex	91-98	95		
	Proconvertin	95-98	97		
	Stuart factor	95-98	97		
	AHF-B	96-98	97		
Elution				Eluate	
	Prothrombin	71-104	87	104-170 ×	
	Proconvertin complex	54-100	79	80-145 ×	
	Proconvertin				
	Stuart factor	76-100	86		
	AHF-B	25-84	61	80-170 ×	
Alcohol precipitation				Precipitate	
	Prothrombin	73-220	112	240-400 ×	
	Proconvertin complex	81-154	119	150-400 ×	
	Proconvertin	113-150	131		
	Stuart factor	49-77	60		
	AHF-B	91-120	102	120-140 ×	
Lyophilization				Lyophilized product	
				Method 1†	*Method 2*‡
	Prothrombin	36-95	66	110-220 ×	300-400 ×
	Proconvertin complex	33-100	77	110-200 ×	210-400 ×
	Proconvertin			200-280 ×	
	Stuart factor			120-140 ×	200-500 ×
	AHF-B			60-160 ×	180-400 ×
Over-all	Prothrombin	40-124	81		
	Proconvertin complex	40-114	81		
	Proconvertin	78-149	99		
	Stuart factor	40-99	72		
	AHF-B	21-87	46		

*Compared to normal plasma as unity.

†Method 1: lyophilization of all that precipitates at 25 per cent ethanol and pH 5.2

‡Method 2: lyophilization of all that precipitates at 25 per cent ethanol and pH 5.2 after a first precipitate at 18 per cent ethanol and pH 6.8 has been discarded.

Further Purification.—In 6 of the 10 lots, attempts were made to purify prothrombin, proconvertin, Stuart factor, and AHF-B further; a first precipitation from the eluate was effected by adjusting the pH to 6.8 and adding cold ethanol to a final concentration of 18 per cent, approximately as Surgenor suggested.[28] By removing this yellow, lipid-rich fraction, which is poorly soluble after lyophilization and whose content of these 4 factors is relatively low, then proceeding under the conditions described in the previous section, it was possible to attain concentrations over that of plasma, per milligram of protein of the coagulant factors, twice those achieved by a single precipitation (compare Methods 1 and 2 in Table I). Thus, for prothrombin, concentrations of 300 to 400 were attained.

Intravenous Injection of the Fraction in Rabbits.—Rabbits weighing 2.6 to 3.2 Kg. were used. Of 12 rabbits studied, 2 were controls which received neither BL-5 (cyclocumarol, Abbott) nor PPB; 2 received only BL-5 and no PPB; and 8 received BL-5 followed by 1 of the 2 lots of PPB tested in vivo. The fraction was injected 4 or 6 days after the rabbits

had received a total of 75 to 100 mg. of BL-5 orally during the first 5 days. Volumes of fraction injected, 1 to 2 ml., were calculated to bring the rabbit plasma levels up to approximately 100 per cent in prothrombin. Oxalated blood samples were obtained from the ear veins. The blood sample collected 10 minutes after the injection of PPB was drawn from the opposite ear from that into which the fraction was injected.

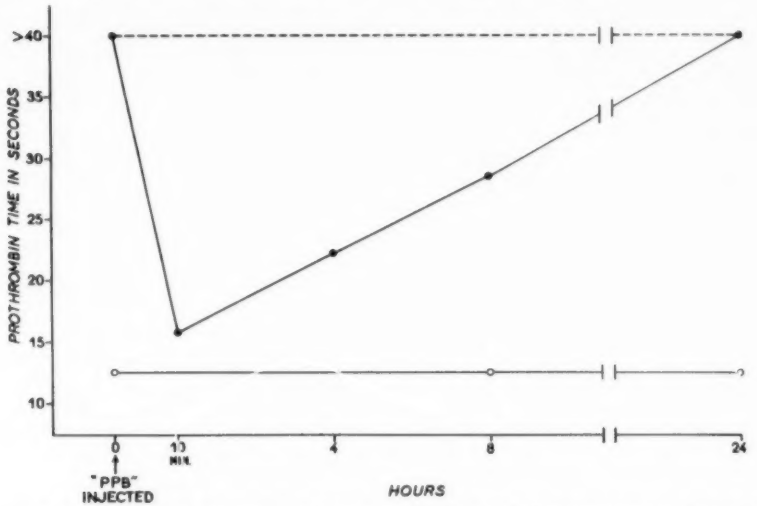

Fig. 1.—Effect of intravenous injection of "PPB" on Quick's prothrombin time of rabbits which have received BL-5 (cyclocumarol). Average of results in 8 injected rabbits. Broken line: uninjected rabbits having received BL-5 alone. Open circles: uninjected rabbits having received no BL-5.

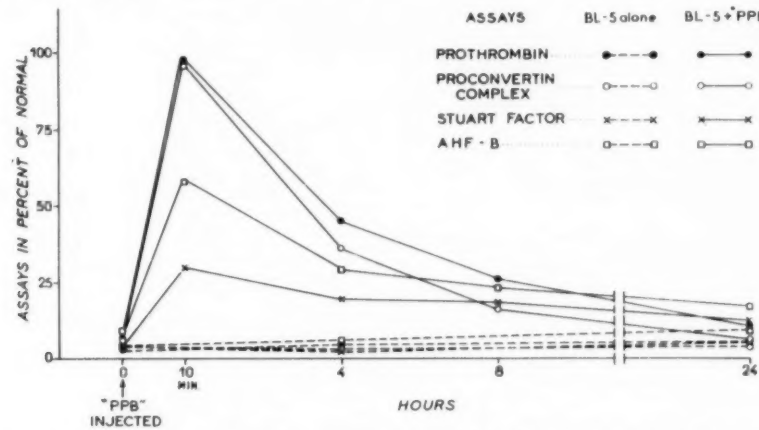

Fig. 2.—Prothrombin, proconvertin complex, Stuart factor, and AHF-B in same experiment as Fig. 1.

Figs. 1 and 2 show the effect of the injection of PPB on the prothrombin times (Quick's one-stage method) and the levels of prothrombin, proconvertin complex, Stuart factor, and AHF-B. The average of all results is represented. At 10 minutes following injection, the

prothrombin time was shortened from over 2 minutes to 15.7 seconds, the normal being 13.0 seconds, and levels of prothrombin and proconvertin complex, initially below 10 per cent, were completely normalized. Corrections of AHF-B and Stuart factor levels, also initially below 10 per cent, were less complete, the peak at 10 minutes averaging 58 and 30 per cent of the normal, respectively. A steep initial decay curve was observed in all 5 test systems; 4 hours after injection the average level in each test system was 49 per cent of the initial level, and 8 hours after injection it was 36 per cent. Correlation between expected and actual initial rise was close for prothrombin and proconvertin complex but less good for Stuart factor and AHF-B.

The rabbits receiving PPB showed no untoward symptoms during the immediate 3-hour period following injection.

Of the 5 rabbits receiving 100 mg. BL-5 over 5 days, 4 died, one of which had received BL-5 alone (not followed by PPB); of the 5 rabbits receiving 75 mg. BL-5 over 3 days, none died. Deaths occurred 3 to 48 hours following injection. Autopsies performed on 3 of the 4 rabbits which died showed extensive hemorrhages in pulmonary parenchyma, pleural cavity, subcutaneous tissue, and mucosa of large intestine. Hemoptysis, epistaxis, and uncontrollable hemorrhage from site of venepuncture were the commonest symptoms before death.

Average rectal temperature of 4 of the injected rabbits mounted to a peak of 0.9° C. above normal 1 hour after injection and had returned to normal by 2½ hours.

The 5 surviving rabbits showed no untoward reactions in the 2-week period of observation following injection of PPB.

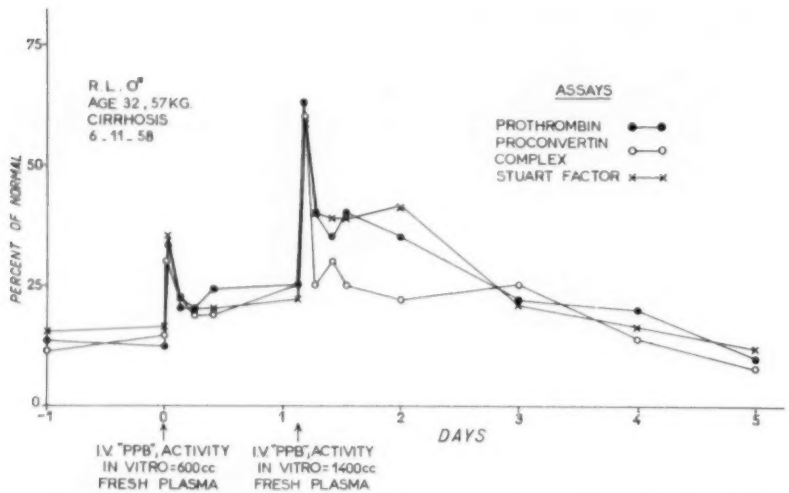

Fig. 3.—Effect of two intravenous injections of sterile "PPB" on plasma prothrombin, proconvertin complex, and Stuart factor levels in a man with chronic severe hepatocellular insufficiency.

Clinical Trial of the Fraction.—Two lots, prepared as described in the paragraph headed Further Purification and sterile-filtered through a Chamberland No. 5L5 porcelain bougie (Paris) before freezing and lyophilizing, were each dissolved in 100 ml. normal saline and intravenously administered 24 hours apart, each over a period of 30 minutes, to a 32-year-old man with chronic severe hepatocellular insufficiency, but without ascites or severe hemorrhagic symptoms (Fig. 3). Preinjection levels of prothrombin, proconvertin

complex, and Stuart factor were 11 to 16 per cent of normal; AHF-B 40 per cent; proaccelerin 50 per cent; platelet count 80,000 per mm.^{3}; plasma thrombin time 45 sec. compared to a normal of 22 sec. The first injection, prepared from 1,500 ml. of normal plasma, had an in vitro activity in prothrombin, proconvertin complex, and Stuart factor, just before injection, equivalent to 600 ml. normal plasma; the patient's levels of these factors were raised from 11 to 16 per cent to 30 to 35 per cent of normal immediately after the infusion was terminated; prothrombin time (Quick) was shortened from 28.3 to 18.9 sec., the normal being 15.5 sec. The second injection, prepared from 2,700 ml. normal plasma, had an in vitro activity in prothrombin, proconvertin complex, and Stuart factor equivalent to 1,400 ml. normal plasma; the patient's levels of these factors were raised from 22 to 25 per cent to 58 to 63 per cent of normal immediately after the infusion was terminated; prothrombin time was shortened from 20.8 to 15.8 sec., the normal being 13.2 sec.; all levels progressively fell, returning to pre-first injection levels only after 4 days. There was no significant effect on the platelet count, plasma thrombin time, or proaccelerin level. The effect on the AHF-B level was equivocal; this is felt to be due to the fact that the preinjection level (40 per cent) was not sufficiently below the lower limit of normal for the test (50 per cent) for an effect of the fraction to be clearly detectable. The two injections were well tolerated; there was no significant effect on the patient's clinical course, temperature, pulse, respirations, or blood pressure during the period of observation.

DISCUSSION

The method of preparation of PPB is similar to that of Surgenor and co-workers[28]; the main difference is the use here of Baker tricalcium phosphate instead of barium sulfate. Use of this adsorbent has the advantage of having a much higher adsorptive capacity per unit weight; of 5 different makes of tricalcium phosphate and two of barium sulfate tested, Baker "Analyzed Reagent" tricalcium phosphate was found to have an adsorptive capacity for the coagulant factors in question, per unit weight, 3 times that of Fisher tricalcium phosphate and 8 to 12 times that of any of the other tricalcium phosphates or barium sulfates tested. The use of such an adsorbent is probably responsible for the somewhat higher yield during elution, makes elution technically easier, and contributes to the purity of the eluate.

Extrapolating the straight portion of the curves in Fig. 2 back to zero time, the half-lives after equilibrium of the human factors injected into rabbits are calculated to be 5 to 6 hours for prothrombin, 3 to 4 hours for the proconvertin complex, and 10 to 11 hours for AHF-B. Stuart factor appears to have a survival curve similar to AHF-B, though levels of this factor were not raised sufficiently high by the PPB to permit an accurate calculation of its half-life. The half-lives after equilibrium of these human factors injected into man, on the other hand, appear to be significantly longer (50 to 55 hours). It is known that these factors, when injected into a heterologous species, induce an antibody response.[38-39] This fact may be related to the more rapid disappearance of these factors from the circulation following heterologous injection than following homologous injection.

In view of the hemorrhagic lesions seen at autopsy in the 3 rabbits examined which died, the fact that one of these had received BL-5 alone without PPB, and the fact that there were no deaths among the rabbits which received the smaller dose of BL-5 followed by PPB, it seems likely that overcoumarinization of half of the rabbits, rather than any toxicity of PPB, was the cause of the fatalities.

Hoag and associates[7] recently reported the intravenous effect of highly concentrated (49 to 88 times normal plasma by volume) human proconvertin (plus Stuart factor?) on 4 patients with congenital hypoproconvertinemia. This product was made from $Al(OH)_3$ adsorption of outdated plasma. Their survival data in man suggest a considerably shorter half-life during equilibrium (75 to 108 minutes) than those presented here (50 to 55 hours).

Frick[37] studied the disappearance curve of stable factor (proconvertin) in man following a single large dose of Warfarin (3-[α-acetonylbenzyl]-4-hydroxycoumarin), and following infusion of normal plasma in individuals whose stable factor was severely depressed by prior administration of Warfarin. He concluded that the half-life of stable factor in normal man ranges between 5.5 and 10.8 hours. The assay system used is probably sensitive to both proconvertin and Stuart factor, however.

The administration of blood, plasma, or serum to patients with deficiencies of one or more of the factors in PPB has generally been disappointing[14, 29-33]; 600 ml. of fresh whole blood given to a series of adults with chronic severe deficiency of prothrombin and proconvertin had almost no effect on the levels of these factors tested during and at intervals following infusion.[34] Results of such injections are in accord with data presented here concerning the rapid disappearance of the injected factors from the circulating blood. High levels of injected factors cannot easily be obtained, short of an exchange transfusion procedure, unless the injected factors are in concentrated form.

To our knowledge, no commercial product exists containing concentrated human prothrombin, proconvertin, Stuart factor, and AHF-B. Behring Werke (Marburg, Germany) markets ACC-76, which is prepared from human plasma and said to be rich in convertin and accelerin. A lot tested by us, dissolved according to instructions, had little proaccelerin activity and 9 times the proconvertin activity of normal plasma. Its effects in congenital hypoproconvertinemia have been definite, although minimal.[8, 35]

It is possible that human plasma fraction PPB contains the virus of homologous serum jaundice if it was present in the pooled plasma used as source material. Until this question is clarified, such a fraction should be used with caution and the size of the plasma pools kept to a minimum.

SUMMARY

1. A method for preparing a human plasma fraction rich in prothrombin, proconvertin (SPCA precursor, factor VII), Stuart factor, and AHF-B (PTC) is described. Final yields after lyophilization were 46 to 99 per cent, being highest for prothrombin and proconvertin. Prothrombin potency 300 to 400 times that of normal plasma per milligram of protein was repeatedly achieved. No thrombic or thromboplastic activity was detected. The lyophilized product was stable over at least 5 months. Sterile filtration of the product through a Chamberland porcelain bougie without significant loss of activity is practicable.

2. A single intravenous injection of this fraction brings the level of prothrombin to about 100 per cent when the dose calculated to do this is injected

into rabbits made deficient (< 10 per cent) in these factors by prior coumarin administration.

3. The expected rise in plasma levels of prothrombin, proconvertin complex, and Stuart factor was also obtained following the intravenous injection of two sterile lots of this fraction in a patient deficient in these factors consequent to severe hepatocellular insufficiency These injections were tolerated without untoward incident.

4. Data suggest that the half-lives in the circulation after equilibrium of human prothrombin, proconvertin complex, and AHF-B injected into rabbits are between 3 and 11 hours. A preliminary clinical trial suggests human prothrombin, proconvertin complex, and Stuart factor when injected in man have a longer half-life (50 to 55 hours).

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Erratum

In the article by Hans Reichard, M.D., and Peter Reichard, M.D., entitled "Determination of Ornithine Carbamyl Transferase in Serum," which appeared in the November issue of the JOURNAL, the third paragraph under Materials and Methods on page 710 should begin as follows: "Serum (0.5 ml.) was incubated with 10 μM (not 1 μM as appeared in the JOURNAL) citrulline carbamyl-C^{14}. . . ."

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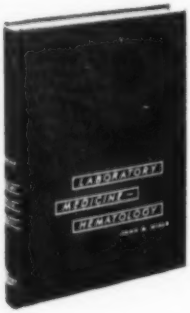

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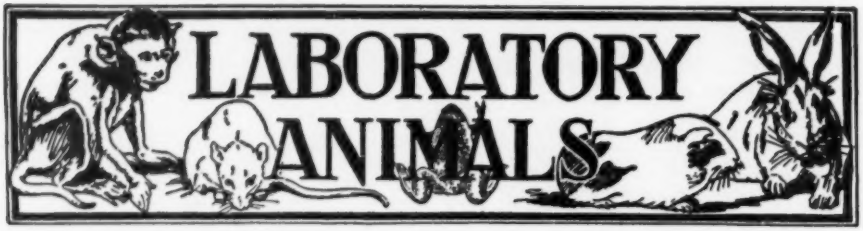

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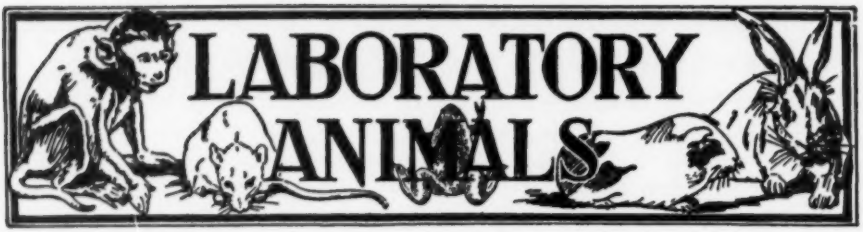

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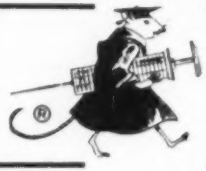

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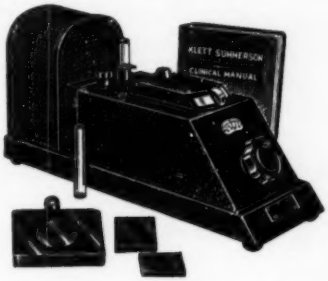

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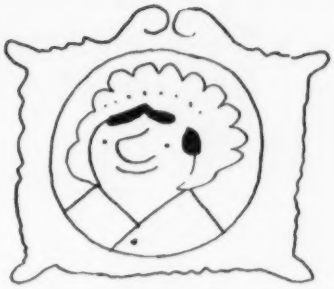

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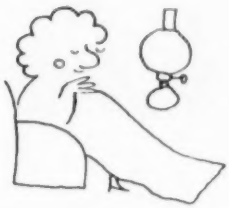

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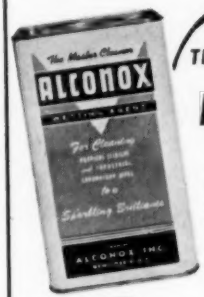

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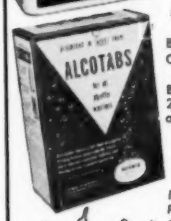

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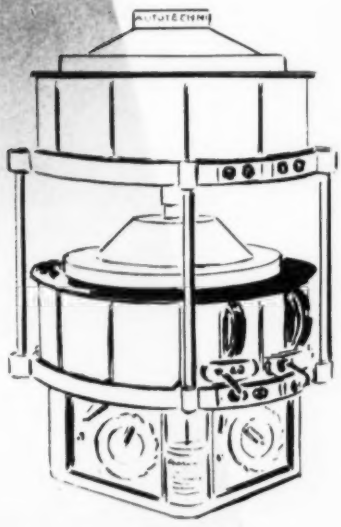

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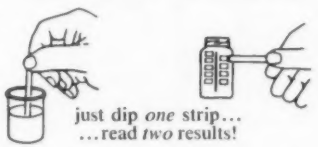

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